MAR 6 – 1933

THE AMERICAN JOURNAL OF PHYSIOLOGY

EDITED FOR

THE AMERICAN PHYSIOLOGICAL SOCIETY

CONTENTS

STUDIES ON THE PHYSIOLOGICAL ACTION OF GLYCEROL ON THE ANIMAL ORGANISM. *Victor Johnson, A. J. Carlson and Adelaide Johnson* 517

MELANOGENESIS WITH SPECIAL REFERENCE TO SULFHYDRILS AND PROTAMINES. *H. J. Ropshaw* 535

ON THE CHEMICAL COMPOSITION OF LYMPH FROM SUBCUTANEOUS VESSELS. *J. William Heim* 553

AN EXPERIMENTALLY PRODUCED PREMATURE SYSTOLIC ARHYTHMIA (PULSUS BIGEMINUS) IN RABBITS. V. FACTORS THAT AFFECT ITS ONSET, DURATION, ARREST AND ALTERATION. *William F. Allen* 559

STUDIES ON THYROGLOBULIN. II. ABSORPTION OF THYROGLOBULIN AND RELATED SUBSTANCES FROM THE ALIMENTARY CANAL. *B. O. Barnes and J. G. Bueno* 570

CHEMICAL OBSERVATIONS ON FLUIDS OF THE SEMINAL TRACT. I. INORGANIC PHOSPHORUS, CALCIUM, NON-PROTEIN NITROGEN AND GLUCOSE CONTENT OF SEMEN AND OF SEMINAL VESICLE, PROSTATE AND SPERMATOCELE FLUIDS IN MAN. *C. B. Huggins and Anna A. Johnson* 574

A NEUROMUSCULAR MECHANISM IN THE DUCTUS EPIDIDYMIDIS AND ITS IMPAIRMENT BY SYMPATHETIC DENERVATION. *F. A. Simeone* 582

OBSERVATIONS ON THE FLOW OF BLOOD IN THE FEMORAL ARTERY OF THE DOG EIGHT TO THIRTY-FOUR MONTHS AFTER LUMBAR SYMPATHECTOMY. *J. F. Herrick, Hiram E. Essex and Edward J. Baldes* 592

THE CAUSE OF MAMMARY DEVELOPMENT DURING PSEUDO-PREGNANCY IN THE RABBIT. *S. A. Asdell and G. W. Salisbury* 595

THE EFFECTS OF PROLONGING THE LIFE OF THE CORPUS LUTEUM IN THE RABBIT BY HYSTERECTOMY. *S. A. Asdell and J. Hammond* 600

ANIMAL ADAPTATION TO ENVIRONMENTAL TEMPERATURE CONDITIONS. *Cordelia Ogle and C. A. Mills* 606

PHYSIOLOGICAL VARIATIONS IN THE GLUCOSE RATIO OF BLOOD AND CEREBROSPINAL FLUID. *David J. Cohn, Abraham Levinson and Frances McCarthy* 613

THE EFFECTS OF SUGAR AND ELECTROLYTE SOLUTIONS ON THE METABOLISM AND IRRITABILITY OF HEART MUSCLE. *Joseph Victor* 620

THE CONTINUED INJECTION OF OESTRIN INTO YOUNG RATS. *Gregory Pincus and N. Werthessen* 631

THE FAILURE OF SYMPATHETIC EXTIRPATION IN THE RAT TO AFFECT THE BASAL METABOLISM OR THE CALORIGENIC ACTION OF ADRENALINE. *M. O. Lee and Z. M. Bacq* 637

THE TIME AT WHICH CASEIN BEGINS TO BE FORMED IN THE BREAST DURING PREGNANCY. *Oran I. Cutler and Julian H. Lewis* 643

ABNORMAL GROWTH OF WATTLES AND TESTES OF COCKERELS AFTER REMOVAL OF COMBS. *G. Davis Buckner, W. M. Insko, Jr. and J. Holmes Martin* 647

A COMPARISON OF THE TIME RELATIONS OF MUSCLES SUPPLIED BY NORMAL AND BY REGENERATED NERVES. *Donald H. Barron* 651

ELECTRICAL POTENTIALS IN SMOOTH MUSCLE. *A Rosenblueth, C. Leese and E. Lambert* 659

THE NATURE OF THE RESPONSES OF SMOOTH MUSCLE TO ADRENIN AND THE AUGMENTOR ACTION OF COCAINE FOR SYMPATHETIC STIMULI. *A. Rosenblueth and D. McK. Rioch* 681

STUDIES ON THE ADRENAL. I. ADRENALECTOMY IN MAMMALS WITH PARTICULAR REFERENCE TO THE WHITE RAT (MUS NORVEGICUS). *W. M. Firor and Arthur Grollman* ... 686

(Continued on cover 4)

VOL. CIII—No. 3

Issued March 1, 1933

BALTIMORE, U. S. A.

1933

Entered as second-class matter, August 18, 1914, at the Post Office in Baltimore, Md., under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 5, 1918.

Made in United States of America

PHYSIOLOGICAL APPARATUS

1. Simple adjustable stand: 24 inches high, total weight 11 pounds. By means of a worm gear, the vertical ½ inch steel-rod can be rotated through 360 deg., chromium plated.....$9.00

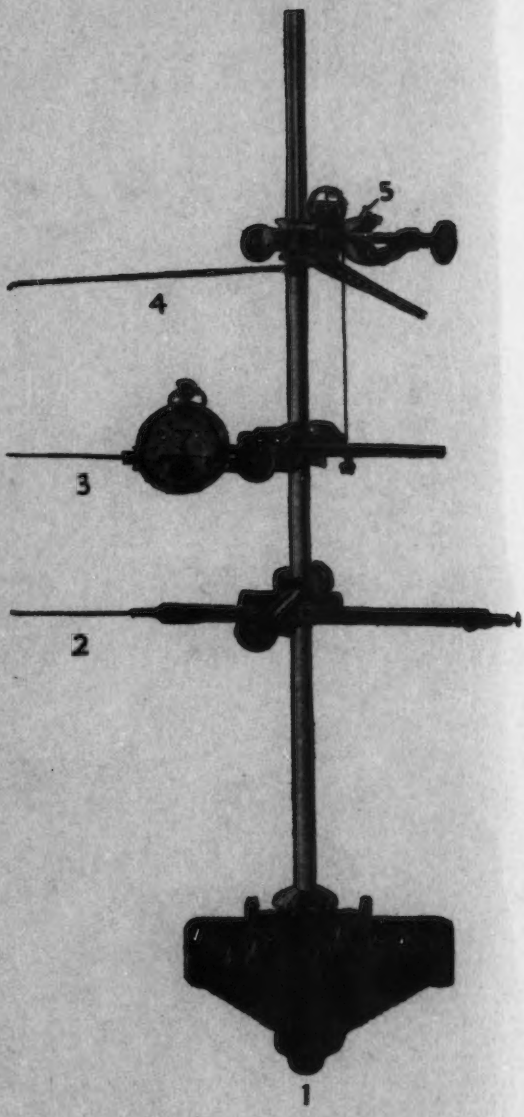
2. Signal Magnet: this instrument consists of a brass tube ⅜ inch in diameter, which contains an electro magnet. The extent of the movement of the writing point can be regulated by adjusting the milled screw cap.....$3.50

3. This time recorder was described by Prof. C. C. Lieb in the Journal of Pharmacology and Experimental Therapeutics (Vol. IX, 1906–17, 237). The second hands of a watch are replaced by toothed wheels which activate a writing level. Time may be recorded in minutes alone, in minutes and seconds, or in minutes and 5 seconds periods.....$10.50

4. A very light but strong lever. One arm consists of a slit brass tubing which holds the writing straw, the other arm when in line with the writing lever, counterbalances it. The load on the lever may be increased by moving the counterbalancing arm around an axis. The yoke is H-shaped, one yoke carrying the writing lever, the other a small pulley, and may be fixed to the supporting rod in any of the six positions.....$8.50

5. This adjustable clamp is compact and strong, it is made of bronze and chromium-plated. The screw clamps may hold a rod in vertical or horizontal positions. The movable half of the instrument can be swung through an arc of 140 deg. by turning a milled screw head.....each $3.75

The whole outfit.....$40.00



Manufactured by **JOSEPH BECKER**

630 WEST 168TH STREET, NEW YORK

THE AMERICAN
JOURNAL OF PHYSIOLOGY

VOL. 103 MARCH 1, 1933 No. 3

STUDIES ON THE PHYSIOLOGICAL ACTION OF GLYCEROL ON THE ANIMAL ORGANISM

VICTOR JOHNSON, A. J. CARLSON AND ADELAIDE JOHNSON

From the Department of Physiology, University of Chicago

Received for publication November 12, 1932

As an integral part of fats, which constitute part of the normal diet of mammals, glycerol is constantly being split off in the gastro-intestinal tract and absorbed by the mucosa. It would therefore seem safe to conclude, a priori, that this substance is innocuous. Yet a number of investigations have indicated that this is not unconditionally true. Glycerol administration has been reported to lead to destruction of red blood cells and hemoglobinuria (Ustimowitsch, 1876; Plosz, 1878; Simon, 1915) even giving rise to anemia (Garibaldi, 1920); albuminuria (Ustimowitsch, 1876) and fever (Plosz, 1878, and others); and in larger doses (8 to 15 grams per kilogram body weight) to tachycardia, vomiting, muscular weakness, convulsions, and even death (Plosz, 1878, Simon, 1921). At autopsy damage to various tissues has been reported by Plosz and also by Piras (1925). It seems not to have been pointed out, however, that the route of administration may determine whether or not glycerol is injurious. Numerous examples could be cited of innocuous and even indispensable materials occurring in the gastro-intestinal tract which kill if injected subcutaneously, intraperitoneally, or intravenously. Examination of the literature with this in mind reveals the fact that it is essentially in instances of parenteral administration of glycerol that injurious consequences have been reported. This is true of the production of hemolysis by Simon (1915), anemia by Garibaldi (1920), tissue damage by Piras (1925), and death by Simon (1921). While Ustimowitsch (1876) employed both oral and parenteral administration in his extended studies, he does not clearly indicate any relation between the route of administration and the injurious effects observed. But it is significant that the protocols he publishes of instances of hemoglobinuria are of cases of intravenous injection. And furthermore in protocols of an experiment on diuresis using the oral route he says:

"der Versuch wurde unterbrochen bevor die Harnrothung in die Regel einzutreten pflegt." It seems legitimate to question whether hemoglobinuria was not the rule only when the glycerol was given otherwise than by mouth. Indeed Arnschink (1887), Leo (1903), and Nicloux (1903) were unable to obtain hemoglobinuria from feeding glycerol.

Also, it is easy to conceive of ingestion of large quantities of glycerol causing serious if not fatal changes through osmotic effects alone especially if the stomach is empty. Plosz (1878) fed dogs as much as 15 grams per kilogram body weight. Ingestion of several hundred grams of table salt or sugar would likewise produce serious changes which would in no wise place these substances in the category of toxic materials.

In the experiments of Long and Vorwald (1930) it was demonstrated that one or more daily intravenous injections of glycerol increased the susceptibility of rats to artificial infection by the tubercle bacillus. These experiments were suggested by the fact that in vitro glycerol seems to have a specific growth promoting effect on the tubercle bacillus (Nocard and Roux, quoted by Frouin and Guillaumie, 1924). Bearing in mind the fact that it has been repeatedly demonstrated that parenteral administration of glycerol may have such serious effects as hemolysis, anemia, and other tissue damage, it would seem highly probable that intravenous injection particularly might decrease the resistance of the animals to any infection, and not at all specifically to tuberculosis. Our dogs frequently displayed marked hemoglobinuria from intravenous injection of glycerol and some even died in a few minutes from a single large injection. It is significant that of the seven rats injected by Long and Vorwald one did die as a result of an injection, and two died of pneumonia. As none of the controls died of pneumonia, the results would equally well lend support to the view that glycerol (intravenously) reduces resistance to pneumonia. These authors also reported a preliminary study in which two rats fed glycerol were compared with controls in their susceptibility to artificially induced tubercle infection. Here, while the results were in the same direction, "the differences in the extent of tuberculosis . . . . were, however, not great . . . ." If these two rats, whose weights are not given, were really "fed with as much glycerol as they would tolerate," as stated, it would not be at all surprising if they were rendered less resistant to any infection.

Because of the rather extensive use of glycerol as a preservative of fruits, meat, and eggs, in pharmaceutical preparations, and even in baking, it seemed desirable to study extensively the effects of ingestion of quantities of glycerol well beyond the maximum which would be taken even if use of the material were extended. Information as to possible ill effects in human beings are most desirable, but before such studies were made extended work was done on rats and dogs.

OBSERVATIONS ON RATS. In three separate growth experiments varying quantities of starch of a standard diet were replaced by equal weights of glycerol. Diet I, the control, consisted of 61 per cent starch, 18 per cent casein, 10 per cent butter, 5 per cent yeast, 4 per cent inorganic salt mixture and 2 per cent agar. The glycerol test diets were identical, except that instead of the 61 per cent starch of diet I, diet II contained 20 per cent glycerol and 41 per cent starch, diet III had 41 per cent glycerol and 20 per cent starch, and diet IV had 61 per cent glycerol and no starch. The food was administered by the method described by Still and Koch (1928), which insures a minimum of scattering of food as well as ingestion by each group of rats of the same amount of food per gram body weight per day. Thus no variation in rate of growth can be attributed to one group eating more food than another. The cages were all equipped with wire mesh false bottoms so that all excreta dropped through. Also, the cages were cleaned and sterilized once a week. The rats themselves were dipped weekly into dilute creosote solution.

Figure 1, of the growth curves of the most prolonged experiment, run 40 weeks on 51 rats, shows that diet III (41 per cent glycerol—20 per cent starch) is at least as adequate as the control diet. It is seen that for the first 20 weeks the glycerol fed animals grew even better than the controls. That this was probably not significantly related to the diets is shown by the fact that when the diets of the two groups were switched at the twentieth week no break in either curve resulted. In two other experiments, one comparing diets I, II and III (30 rats run 25 weeks),[1] and another comparing diets I and III (10 rats run 21 weeks) the growth curves ran very much closer together. However, it is clearly evident that when all the starch is replaced by glycerol, which then constitutes 61 per cent of the total diet (diet IV), growth is greatly impaired. The rats on this high glycerol intake also appeared abnormal, for their coats lacked the smooth gloss of the controls, and they were less active.

Figure 1 also illustrates the fact that the impaired growth of these diet IV rats was not due to any toxic effect from the glycerol. For at the seventh week, when starch was added to the diet of 8 rats in the proportion of 6 grams of starch for every 10 grams of diet IV, they immediately resumed normal growth despite an unchanged daily glycerol intake. On the other hand, the nine rats not receiving this additional starch did not gain weight. At the fifteenth week a switch in the diets of these two groups confirmed the preceding findings, for again the simple addition of starch induced normal growth. At the twenty-sixth week diet IV was again resumed in all seventeen animals in this group, and the growth curves flattened out. Except, then, for a short period of growth at the

[1] This experiment was carried out by Dr. S. Szurek.

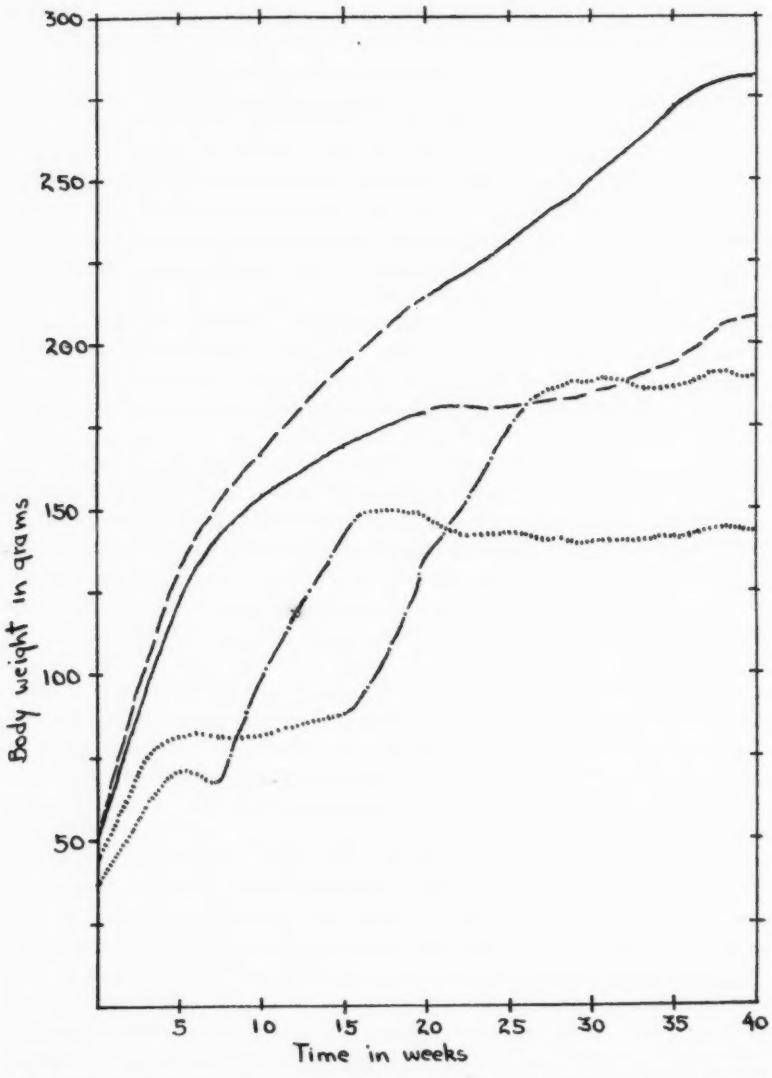


Fig. 1. Growth curves of 51 rats fed diets in which varying quantities of starch were replaced by equal weights of glycerol.

Solid line: diet I, 61 per cent starch, no glycerol.

Broken line: diet III, 20 per cent starch, 41 per cent glycerol.

Dotted line: diet IV, no starch, 61 per cent glycerol.

Dot-and-dash line: diet IV plus 6 grams of starch for each 10 grams of diet IV fed.

beginning of the experiment diet IV was inadequate to support growth when fed in quantities equal by weight to the growth promoting control diet. Diet IV plainly did maintain previously attained body weight, however. These findings indicate that failure to grow probably resulted not

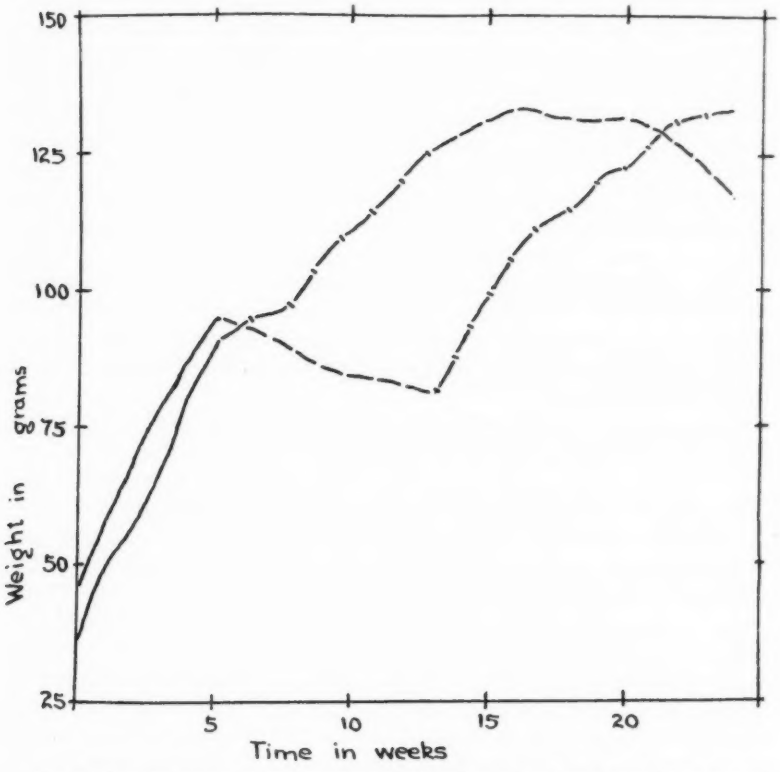


Fig. 2. Growth curves of 10 rats showing the effect of adding glycerol to a quantitatively inadequate diet:

Solid line: two groups of rats on a qualitatively and quantitatively adequate diet.

Broken line: five rats on a reduced intake of the same diet.

Dot-and-dash line: five rats on the reduced intake *plus* 12 grams of glycerol for every 10 grams of the normal diet fed.

from any toxic effect of the glycerol, but rather from inadequate quantitative intake. Confirmatory to this view is the fact that this group seemed unusually hungry, becoming very excited when food was being administered, fighting each other for a first chance at the food and ravenously disposing of it. (The reason for not feeding this group all it would eat

was that the rats were not fed ad libitum, but each group was fed proportionally to body weight as described above. Only in this way could a fair comparison of the diets be made.) Extremely rarely did this group fail to eat all it was fed. That there was no difficulty in getting the rats to eat diet IV was surprising, for the sickening sweet taste of even one meal containing 61 per cent glycerol is extremely distasteful to a human being.

To obtain data on the effects on growth from addition of glycerol to the diet, instead of replacing starch with glycerol, an experiment was performed whose results are shown in figure 2. Ten rats were fed the same control diet for five weeks, when the quantity fed to 5 was reduced just below the minimum required to maintain their weight. At the same time the remaining 5 rats received exactly the same diet qualitatively and quantitatively, plus 12 grams of glycerol for every 10 grams of the control diet. The latter group continued to grow at the normal rate. Thus not only was this large amount of ingested glycerol (over 50 per cent of the total diet) not toxic, but it actually furnished sufficient calories to make the difference between bare maintenance of weight or less, and normal growth. A reversal of the diets of the two groups at the thirteenth week fully substantiated this conclusion.

Theoretically, if combustion of the glycerol were complete the caloric yield would be slightly greater than that of an equal weight of glucose. It is probable, however, that some of the ingested glycerol is excreted in the urine either as glycerol (Nicloux, 1903) or in modified form (Plosz, 1878, and others). But the experiments described amply demonstrate that within extensive limits glycerol compares favorably with carbohydrate as a source of energy. That a large proportion of ingested glycerol is converted to sugar has been repeatedly shown in studies of liver glycogen formation (Luchsinger, 1874; Catron and Lewis, 1929, and others) and blood sugar, both on experimental animals (Voegtlin, Dunn, and Thompson, 1925a) and in humans (Ferber and Rabinowitsch, 1929). Estimates have ranged from 40 per cent conversion to glucose (Thomas, 1924) to 100 per cent (Chambers and Deuel, 1925) in diabetes. Chambers also found glycerol to be anti-ketogenic. It has further been shown that glycerol can furnish adequate protection against otherwise lethal doses of insulin in rats (Voegtlin, Dunn, and Thompson, 1925b) and also that in doses of 5 grams per kilogram, it exerts a protein sparing action in starving dogs (Starack, 1931).

Twenty-seven acute experiments were also performed in which under light ether anesthesia doses of glycerol ranging from 7 to 18 grams per kilogram were administered by stomach tube to 11 rats starved 24 hours. In no case were ill effects observed in the ensuing 24 hours.

Comparison of reproductive activity was made on the three groups receiving diet I (61 per cent starch; no glycerol), diet III (20 per cent starch;

41 per cent glycerol) and diet IV (no starch; 61 per cent glycerol), whose growth curves are given in figure 1. The number of pregnancies, young born, and young weaned were observed. Because the number of females in each group was not the same, reproductive activity is expressed not in terms of pregnancies, etc., per group, but per equal numbers of adult female rat weeks. The number of adult females in a group multiplied by the number of weeks these were housed with adult males on the same diet gives the number of adult female rat weeks. After 275 such rat weeks there were 22 pregnancies, 150 young born, and 74 young weaned in the control group, compared with 16 pregnancies, 101 born and 68 weaned in the glycerol rats. It is seen that although the number of young born to control females is considerably greater than the number born to the glycerol fed rats at the end of the experiment, the number of young weaned by the controls is only slightly greater. Furthermore, at the earlier period of 200 rat weeks all three criteria of reproduction indicate greater activity by the glycerol fed rats than by the controls. On the whole, reproduction seems not to have been hampered by substitution of starch by glycerol to a point where the latter comprises 41 per cent of the total food intake. However, no pregnancies occurred in the diet IV animals, receiving 61 per cent of their diet as glycerol. This was to be expected from the undernourished and debilitated condition in which the amounts of this diet fed kept them.

It was noticed at once that the glycerol fed rats drank more water than the controls. Measurements showed that per unit body weight diet III animals drank 30 per cent more water than the controls, and diet IV rats drank 230 per cent more than the controls. Reference to this observation will be made later, when the subject of diuresis is discussed.

At the close of the growth experiment run on 30 rats for a period of 25 weeks, the rats were killed and autopsied, and histological preparations made of the kidney, liver, and intestine. No abnormalities were found. Piras (1925) had reported congestion, small hemorrhages and cell degeneration of the liver, intestine, and kidneys as a result of small daily doses of glycerol administered over several weeks. Again, significantly enough, Piras used the subcutaneous route, which seems not to be as innocuous as the oral route.

Observations on dogs. The studies were further extended on dogs. Three pairs were used, each pair being litter mates. The control diet, fed to three dogs, consisted of 24.5 per cent protein, 52 per cent carbohydrate and 7 per cent fat, obtained from ground lungs,[2] white bread,[3] and

[2] Analysis: 17.0 per cent protein, 4.0 per cent fat, by Moulton (1922).

[3] Analysis: 7.1 per cent protein, 1.2 per cent fat, 52.3 per cent carbohydrate, by Atwater and Bryant, from Lusk (1917).

whole milk,[4] and also 2.5 per cent yeast and 14 per cent bone meal. After this content of bone meal was arrived at the dogs never showed diarrhea, except for the occasional instances of short duration which may occur in any dog. The glycerol test diet, fed the three litter mates of the controls contained 35 per cent glycerol and 17 per cent carbohydrate as against the 52 per cent carbohydrate of the control diet. Glycerol replaced the carbohydrate of bread, and the protein of bread which was also thus withdrawn was replaced by gluten flour.[5] Control and glycerol diets contained equivalent amounts of meat (lung), milk, yeast, and bone meal. Of course the quantities fed each animal were in proportion to his body weight, so that at the end of the 50 weeks of experimentation each animal had received quantitatively and qualitatively the same food as any other animal, assuming 1 gram of glycerol equivalent to 1 gram of carbohydrate. The dogs were fed separately, but at all other times were allowed to mingle freely in a relatively large enclosed portion of the laboratory. Again it was found that the sweet taste of glycerol seemed in no wise to impair the appetite of the dogs, as had also been true of the rats.

Figure 3 shows the growth curves, commencing with the fifth week, when the puppies, previously weaned on milk and bread, were put on the experimental diets. Until the 36th week the two curves run very close together. At that time all diets were reduced in proportion to from 50 to 80 per cent of the previous intake to see whether at a caloric intake of bare maintenance level, some difference in the two diets might appear. In the ensuing 14 weeks the controls maintained their weight, whereas the glycerol dogs lost an average of 1.8 kgm., or 16 per cent of their previously attained weight. This again probably signifies that glycerol in such large quantities (35 per cent of the total food intake) is not calorically equivalent to glycose. It might be argued that with an adequate intake of other food materials the dogs were in an excellent enough physical condition to withstand a possible toxicity of glycerol, which only displayed itself in the loss in weight following reduction of the total food intake at the 36th week. In answer to this, reference may be again made to the rat experiment plotted in figure 2, in which animals receiving barely enough of a normal diet to maintain body weight were suddenly made to resume growth by the addition of sufficient quantities of the "possibly" toxic glycerol.

The dog experiments made possible a study of the previously mentioned contentions that glycerol administration produces albuminuria, red blood cell destruction, hemoglobinuria, and anemia made by Ustimowitsch (1876), Plosz (1878), Simon (1915), and Garibaldi (1920). It is recalled that

[4] Analysis: 3.2 per cent protein, 3.8 per cent fat, 5.0 per cent carbohydrate from Lusk (1917).

[5] Analysis: 81.0 per cent protein, 1.0 per cent or less fat, 4.0 per cent carbohydrate by Chicago Dietetic Supply House, manufacturers.

Simon and Garibaldi used parenteral administration, that Ustimowitsch does not clearly indicate which route was used when hemoglobinuria appeared, and that the production of hemoglobinuria upon oral administration could not be confirmed by Nicloux (1903) or Leo (1903). The erythrocyte counts for the two groups ran very nearly parallel, crossing and recrossing several times. The lowest red cell count observed in any

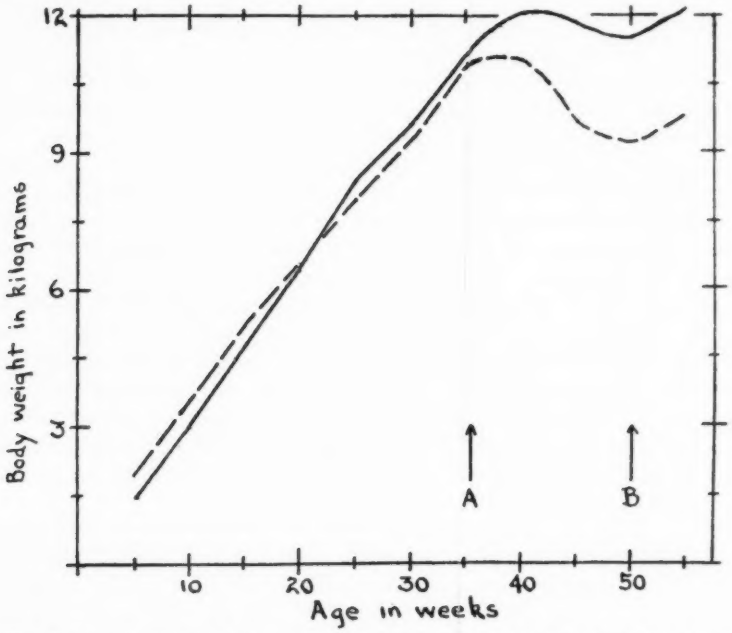


Fig. 3. Growth curves of dogs showing the effect of replacing starch by glycerol in the diet.

Solid line: weights of dogs on a diet containing 52 per cent carbohydrate, no glycerol.

Broken line: weights of dogs on a diet containing 17 per cent carbohydrate, 35 per cent glycerol.

At *A*, total daily intake of all dogs reduced.

At *B*, previous intake resumed.

glycerol dog was 5.4 millions as against a low of 5.3 millions in one of the controls.

Ustimowitsch and others have asserted that glycerol is hemolytic in vitro as well as in vivo. It may be readily demonstrated that this is a partial truth. For while an iso-osmotic solution of glycerol in distilled water will hemolyze red cells as readily as will distilled water, an iso-

osmotic solution of glycerol in 0.9 per cent NaCl does not do so. It is common knowledge that an identical experiment can be performed using urea instead of glycerol, and both exeriments are to be explained by the fact that the red cell "membrane" is freely permeable to both urea and glycerol. Consequently these materials fail to exert any osmotic effects on the red cells and exposure of the cells to iso-osmotic solutions of glycerol or urea in distilled water is practically equivalent to exposing them to distilled water. Simon (1915) recognized that a 1.0 per cent solution of NaCl protects erythrocytes from hemolysis by glycerol.

Examination of the urine of each dog for hemoglobin and albumin was done repeatedly from the 13th to the 45th week of the experiment and in no instance was hemoglobin or albumin detected even in traces. The analyses were made on urine samples collected from 1 to 24 hours after feeding. In addition to this, 71 acute experiments on 22 other dogs were done, in which the glycerol was given per os either with a lung-bread-milk meal, or separately by stomach tube in doses which ranged from 2 to 4 grams per kilogram. Eight of these experiments were on 6 dogs which had been starved 18 to 24 hours. However, the emetic action of glycerol when administered to dogs on an empty stomach, described by Plosz (1878), often rendered it impossible to give the material by stomach tube without first depressing the animal somewhat with morphine and atropine subcutaneously. It is noteworthy that when an emetic dose of glycerol was given by mouth along with lung, bread and milk, no emesis occurred. In none of the 71 tests were albumin or hemoglobin detected in the urine.

There are two reasons for having repeated so frequently these urinalyses on many dogs. In the first place, early in the work one dog not included in the above group did show an albuminuria after a dose of 2 grams of glycerol per kilogram body weight in 50 per cent solution given per os on an empty stomach. Repeated attempts on the same animal failed to elicit a similar response. In the light of the failure to find albumin in the many other acute experiments performed, as well as in the repeated examinations of the urine of the chronic dogs, it would seem justifiable to conclude that in the single exceptional instance of albuminuria (impossible to confirm later) some other significant factor besides the glycerol ingestion was involved. That benign albuminuria may occur from a variety of causes has been repeatedly demonstrated. Perhaps the most extensive study, made by Maclean (1919), showed that 5 per cent of 50,000 normal subjects showed albuminuria in the morning and that this percentage increased with exercise.

In the second place, it was found possible to obtain not only albuminuria but also hemoglobinuria by parenteral glycerol administration. In two intra-peritoneal injections of 3.0 grams of glycerol per kilogram in one dog,

hemoglobinuria occurred once. In 14 subcutaneous injections of 2.5 to 3.0 grams per kilogram in 8 dogs, albuminuria occurred twice and hemoglobinuria three times. The urine studies bring out very forcibly that whereas relatively small doses of glycerol parenterally injected may be toxic, much larger doses per os are innocuous especially if this large dose is taken with other food material. For the three dogs fed the glycerol test diet received over 9.0 grams of glycerol per kilogram per day for nearly a year, and yet suffered no demonstrable ill effects.

In the glycerol fed dogs there was observed the same increased water intake previously noticed with rats. Volume measurements of urine showed that the glycerol animals secreted approximately 5 times as much

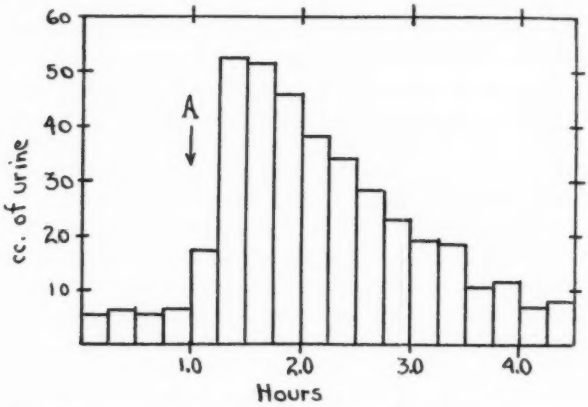


Fig. 4. Diuretic action of glycerol. Each column represents the volume of urine secreted by both kidneys in a 15 minute period. At *A* 2.5 grams of glycerol per kilogram body weight was administered by stomach tube. Figures are averages of 8 experiments on 2 dogs with bladder fistulae.

urine as dogs on the control diet, in equal periods of time. That the increased urine output was not secondary to the increased water intake was readily demonstrated by giving glycerol to dogs having no access to water. Figure 4 shows the results of administration by stomach tube of 2.5 grams of glycerol per kilogram to dogs with bladder fistulae. It is seen that in the first 15 minutes following ingestion the rate of urine flow is almost trebled, and in the second 15 minutes it is increased by nearly nine times. The diuretic action of glycerol had already been noted by Ustimowitsch (1876) and others. Cugusi (1922) states that intravenous injection in small doses produces diuresis, but that larger doses inhibit urine secretion. This inhibition was noticed in our experiments only when the intravenous dose of glycerol produced a marked lowering of blood pressure at the same time.

Characteristically even a small dose of glycerol intravenously produces a transient fall in arterial pressure, during which period the urine flow may be diminished.

The mechanism of the diuresis is probably the same as that responsible for increased urine output in hyperglycemia and glycosuria, for despite the difficulty and uncertainty of estimating glycerol in the urine, there is an agreement by observers using different techniques, that glycerol ingestion leads to the speedy appearance of some of the material in the urine, possibly in modified form (Plosz, 1878; Nicloux, 1903; and others).

The rapidity of the onset of the diuresis suggests that glycerol may be absorbed from the stomach. Experiments on the absorption of glycerol indicated that while some of the material is absorbed from the stomach, about 50 per cent more is absorbed from the intestine in the first two to three hours after ingestion. These experiments (twelve in number) are subject to criticism, however, for the animals were anesthetized, and both the stomach and a loop of intestine were tied off. Also, the method of estimation of the unabsorbed glycerol was the crude one of measuring the volume of fluid remaining after evaporation of the water, glycerol having a boiling point considerably above that of water.

The contention of Plosz (1878) and Arnschink (1887) that glycerol ingestion leads to increase in body temperature was also tested. Numerous observations were made on the 3 chronic glycerol dogs, as well as on 13 other acute dogs fed a meal of meat-bread-milk plus 35 per cent glycerol. Readings were made at feeding, 1 hour later, and 8 hours after feeding. In no case was there an elevation of body temperature.

Throughout the period of observation the condition of the animals was good, except for an ailment early in the experiment which affected controls and glycerol fed animals alike. There was no consistent difference in the behavior of the two groups. Careful autopsy revealed no gross differences in the two groups. Microscopic studies on the stomach, intestine, lung, spleen, kidney and liver revealed normal organs. Formalin fixation and fat stains of the liver revealed no degeneration. Thus there is again a failure to confirm the findings of cell degeneration reported by Plosz (1878) and Piras (1925), the latter worker at least, having employed the subcutaneous route of glycerol administration.

Observations on man. The work so far carried out seemed to warrant continuation of the experiment on the human species. Fourteen graduate students in physiology (10 men, 4 women) served as subjects. For a period of 50 days each subject drank 30 cc. of 95 per cent glycerol with orange juice after each of the three daily meals.[6] Preceding and following

[6] After some experimentation in disguising the sweet taste of glycerol by taking it with coffee, milk, or lemon juice the following method of ingestion was adopted. A layer of orange juice was floated on about 35 cc. of glycerol. Immediately following

this period were ten day control periods. No rigid control of the diet was attempted, each subject eating his customary diet throughout, with these exceptions: each day, in the control as well as glycerol ingestion periods the juice from three oranges was drunk; also any tendency to reduce the intake of normal foods during the glycerol ingestion period was to be avoided, the aim being to test the effects of addition of glycerol to a normal diet. The experimental period fell entirely within a school quarter, so that all subjects were at work normally, and no vacation periods interrupted.

The daily ingestion of 90 cc. or about 110 grams of glycerol amounted to from 1.3 grams to 2.2 grams per kilogram body weight, depending upon the weight of the subject. If all the glycerol ingested were utilized it would yield theoretically about 440 calories per day, which would be at least one tenth of the normal caloric intake of a student.

There was a slight tendency toward an increase in body weight during glycerol ingestion. At the end of the glycerol period five people had gained from 1.5 to 4 pounds and 4 had lost from 1 to 4 pounds, four remaining practically unchanged.

Both red and white blood cell counts and hemoglobin estimations (Sahli) were made twice during each control period and 6 times during the glycerol period, on each subject. The results are summarized in table 1. It is seen that there are no significant changes in the blood picture, which extends and completely substantiates the previous findings in dogs.

Analyses of urine samples were made on each subject twice during each control period and from 5 to 7 times during the glycerol period. Reducing material was found in 12 per cent of 80 analyses of urine from the glycerol period as contrasted with 4 per cent of 47 samples taken in control periods. This may be another manifestation of the conversion of glycerol to sugar, evidence for which has been briefly reviewed above.

Hemoglobin was never found in the urine of these subjects. A trace of albumin was detected once in the glycerol period (subject J. N., female) and twice in the second control period (D. C., female, and R. S., male). These transitory instances of albuminuria are again probably cases of benign albuminuria. The numerous causes for the appearance of this phenomenon are reviewed by Fishberg (1931).

Glycerol ingestion has been stated to give rise to increased uric acid excretion. Rosenfeld (1924) found that glycerol per os in man led to an increase in uric acid excretion from 580 to 695 mgm. per day. Lewis and

---

the rapid drinking of this, leaving about 5 cc. of the viscous material adhering to the sides of the tumbler, a swallow or two of orange juice from another glass was taken. Thus orange juice immediately preceded and followed the glycerol, and there was little to be tasted except the orange juice.

Corley (1923) followed the hourly uric acid excretion after ingestion of 50 grams of glycerol in subjects on a purine-free diet. Calculating from the figures of their experiment in which the increase in uric acid excretion was greatest, one obtains the result that ingestion of 50 grams of glycerol gives rise to an added uric acid excretion of 92 mgm. This would increase the 24 hour excretion calculated from their figures, from 513 to 606 mgm. In two of our subjects duplicate determinations of the uric acid content of 24 hour urine samples were made twice in each control period and 5 times during glycerol ingestion. The results, expressed as averages, show that the excretion was 523 mgm. per day before, 512 mgm. during, and 506

TABLE 1

Table showing the effect on the blood picture of ingestion of 110 grams of glycerol daily in man

	NUMBER OF SUBJECTS F: FEMALE M: MALE	CONTROL 10 DAYS AVERAGE OF 2 DETERMINATIONS	INGESTION 50 DAYS AVERAGE OF 6 DETERMINATIONS	CONTROL 10 DAYS AVERAGE OF DETERMINATIONS
Red blood cell counts in millions per cu. mm.	4 f.	5.0	4.9	5.1
	10 m.	5.7	5.8	5.6
	All 14	5.5	5.6	5.5
	M.L.* m.	6.3	5.7	6.1
	K.R.† m.	5.1	5.6	4.9
Grams of Hb. per 100 cc. blood (Sahli)	4 f.	15.5	16.4	14.7
	10 m.	17.0	17.6	17.8
	All 14	16.6	17.2	16.9
	R.S.* m.	17.4	16.9	18.2
	F.G.† f.	16.2	17.6	15.1
White cell count in thousands	All 14	7.3	7.1	7.0
	F.G.* f.	8.1	5.3	7.1
	J.H.† m.	5.4	6.9	4.3

*Shows greatest decrease, † shows greatest increase during glycerol ingestion.

mgm. after the glycerol ingestion period. It must be borne in mind that there was no rigid control of food intake in these experiments.

Until more is known of the metabolic processes involved in the production of the uric acid of the urine, the implications and significance of increases of the order of magnitude obtained by Rosenfeld and by Lewis and Corley cannot be stated. However, the interest of one group of workers in this phenomenon centers about the possibility of formation of uric acid urinary calculi. Rosenfeld (1924) reports that glycerol feeding leads to the appearance in the urine of uric acid crystals, concretions, and sediment, resulting from the alleged capacity of glycerol to precipitate the uric acid of the urine as crystals of sediment. On the other hand Hansen and Kamm

(1926) assert that uric acid and sodium urate are soluble equally well in water, or glycerol, or mixtures. They even believe that glycerol is useful as a therapeutic agent in ridding the bladder of uric acid stone. It does this by virtue of the fact that calcium oxalate, which they say constitutes the "framework" of even uric acid stones, is more soluble in glycerol than in water. Thus the appearance in the urine of uric acid sediment would be due to the disintegration of preëxisting stones rather than to the formation of new stones. These workers add that the sedimentation time of ground-up uric acid crystals is much prolonged in urine or serum to which glycerol has been added. But without drawing final conclusions as to any such possible beneficial action of glycerol, it does seem that there is little or no justification for the assertion that glycerol ingestion favors the formation of urinary calculi.

Urine output comparisons were made on two subjects to determine whether the diuresis observed in the rats and dogs occurs in man. The average 24 hour secretion was 1450 cc. before, 1380 cc. during, and 1300 cc. after glycerol ingestion. This absence of diuresis is probably due to the smaller quantities of glycerol ingested by man. Lewis and Corley (1923) obtained similar results. Of their five subjects receiving 50 grams of glycerol three showed no diuresis. Correlated with this lack of diuresis it was found in our observations that the specific gravity of the urine remained practically constant.

On six subjects curves were made of the variations in body temperature throughout the day. Examination of curves in which average figures are charted reveals the fact that not only is there no consistent rise in body temperature, but also that the curves remain unaltered in shape. The results from each individual are similar to this, each of the three curves taken before, during, and after glycerol ingestion, crossing and recrossing one another repeatedly.

Basal metabolic rate determinations yielded average figures of 66.1 calories per hour before, 61.9 during, and 64.5 after glycerol ingestion in 3 subjects whose average "normal" rate was 65.1 calories per hour. It is seen that the average rate during glycerol ingestion is just under (by 5.2 per cent) the control rate. This decrease was mostly due to the fall in rate of subject D. C., who normally shows a definite plus basal rate. The tendency in her case for the rate to approach the normal rate (for her) helped bring the averages down to a point below the normal rate for the group. Subject F. G. had a sub-normal rate throughout. Rate variations of the order of magnitude found are probably insignificant. These results are in conformity with those of McCann and Hannon (1923) who showed that in the two hours following ingestion of 40 to 100 grams of glycerol three subjects (one diabetic, two normal) showed a slight increase in oxygen consumption, and two (one diabetic, one normal) showed a slight

decrease. In other words, no consistent change resulted from glycerol ingestion.

No dietary experiment on human beings would be complete without a study of the effect of the diet on the activity of the large intestine. Neither rats nor dogs had shown any changes in bowel activity, but the most dependable information was obtained from the human studies. A record of the number of bowel movements per day was kept by each subject. Average figures of the results showed that no significant change occurred in any subject, and the group as a whole remained fairly constant, there being an average of 1.6 bowel movements per day before, 1.5 during, and 1.4 after the glycerol ingestion period. In three subjects the irregularity in number of movements from day to day was slightly greater during the glycerol period than during the control periods.

Even more important than the number of daily bowel movements is their consistency. An analysis of the distribution of the stools into the 5 categories of loose, soft, normal, firm, and hard showed that there was a slight tendency toward softer stools and a very slight decrease in firmer stools during glycerol ingestion. While this displays itself in the records, the changes were not marked enough for any of the subjects to list a tendency to looser stools amongst the effects they themselves had observed, except for one individual who thought that glycerol ingestion relieved a slight tendency toward constipation.

The subjective effects noticed included: increased thirst occasionally (2 subjects), an occasional sensation of warmth in the stomach after ingestion, especially in those exceptional instances when the glycerol was taken not with a meal, but on an empty stomach (4 subjects), and even "a feeling of particular well being and exceptional freedom from colds" (1 subject). There were no reports of the sleeplessness, talkativeness and excitement resembling alcohol intoxication described by Lewin (quoted by Schmey, 1909) from ingestion of 40 to 60 grams of glycerol daily. Schmey also quotes from Battenstein, describing a case of glycerol addiction, in which the victim took from 100 to 1000 grams of glycerol daily, the latter amount for a month. Thus far no signs of addiction have displayed themselves in our subjects.

SUMMARY

1. In studies of replacement of carbohydrate by glycerol in the diet of rats (40 weeks) to a point where glycerol constituted 41 per cent by weight of the total food intake, and in the diet of dogs (50 weeks) until it amounted to 35 per cent of the intake, normal growth occurred. Reproduction in rats was unaffected.

2. Addition of glycerol to a rat diet qualitatively adequate but quantitatively just sufficient for maintenance of body weight caused a resump-

tion of normal growth. Addition of 110 grams of glycerol per day (50 days) to an adequate diet in man produced a slight tendency toward increased body weight.

3. Ingestion by growing dogs of 9 grams of glycerol per kilogram per day for nearly a year did not change the red blood cell count. Ingestion by man of 110 grams of glycerol per day for 50 days did not affect the red or white cell counts or the hemoglobin content of the blood.

4. In neither man nor dogs was albuminuria or hemoglobinuria produced from glycerol taken orally, although both these phenomena were produced in dogs upon parenteral administration.

5. Glycerol per os in relatively large quantities exerts a diuretic action in dogs and rats, but in the quantities fed to man it lacked this effect.

6. The body temperature of man and dogs was unaffected by glycerol ingestion.

7. No adverse subjective effects in man and no changes in the general appearance and behavior of rats and dogs throughout the observation periods, resulted from glycerol ingestion in the quantities used.

8. In man the basal metabolism and uric acid excretion were not significantly affected.

9. Activity of the large intestine of man as regards daily number and consistency of stools, was unaffected by the quantities of glycerol fed.

10. No gross or microscopic pathology was detected in rats and dogs reared (for nearly a year) on diets of high glycerol content.

BIBLIOGRAPHY

ARNSCHINK. 1887. Zeitschr. f. Biol., xxiii, 413.

CATRON AND LEWIS. 1929. Journ. Biol. Chem., lxxxiv, 553.

CHAMBERS AND DEUEL. 1925. Journ. Biol. Chem., lxv, 21.

CUGUSI. 1922. Arch. di Farm. Sper., xxxiv, 150.

FERBER AND RABINOWITSCH. 1929. Amer. Journ. Med. Sci., clxxvii, 827.

FISHBERG. 1931. Hypertension and nephritis. Lea & Febiger, Philadelphia, p. 256.

FROUIN AND GUILLAUMIE. 1924. Compt. Rend. d Seances de la Soc. de Biol., xc, 731.

GARIBALDI. 1920. Biochim. e Terap. Sper., vii, 52.

HANSEN AND KAMM. 1926. Biochem. Zeitschr., clxxiii, 327.

LEO. 1903. Pflüger's Arch., xciii, 269.

LEWIS AND CORLEY. 1923. Journ. Biol. Chem., lv, 373.

LONG AND VORWALD. 1930. Amer. Rev. Tuberculosis, xxii, 636.

LUCHSINGER. 1874. Pflüger's Arch., viii, 289.

LUSK. 1917. Elements of the science of nutrition. Saunders & Co., Philadelphia, pp. 580 and 581.

MACLEAN. 1919. Medical Research Commission, Special Report Series, no. 43, London.

MCCANN AND HANNON. 1923. Johns Hopkins Hosp. Bull., xxxiv, 73.

MOULTON. 1922. Univ. Mo. Agric. Expt. Sta. Res. Bull., no. 55.

NICLOUX. 1903. Journ. de Physiol. et de Path. Gen., v., 827.

PIRAS. 1925. Arch. di Farm. Sper., xl, 251.

PLOSZ. 1878. Pflüger's Arch., xvi, 153.
ROSENFELD. 1924. Klin. Wochenschr., iii, 1908.
SCHMEY. 1909. Deutsch. Med. Wochenschr., xxxv, 1706.
SIMON. 1915. Arch. di Farm. Sper., xx, 120.
1921. Arch. di Sci. Biol., ii, 78.
STILL AND KOCH. 1928. This Journal, lxxxvii, 225.
STRACK. 1931. Tagung der Deutschen Physiologischen Geseslichaft, Berichte uber die Gesamte Physiologie, lxi, 376.
THOMAS. 1924. Johns Hopkins Hosp. Bull., xxxv, 201.
USTIMOWITSCH. 1876. Pflüger's Arch., xiii, 453.
VOEGTLIN, DUNN AND THOMPSON. 1925a. Journ. Biol. Chem. lxiv: 639.
1925b. This Journal, lxxi, 574.

MELANOGENESIS WITH SPECIAL REFERENCE TO SULFHYDRILS AND PROTAMINES

H. J. ROPSHAW

From the Laboratory of Experimental Medicine, School of Medicine, Western Reserve University

Received for publication November 14, 1932

The dopa theory of melanogenesis, as elaborated by Bloch and his collaborators, has stimulated investigations not only on the specificity of dioxyphenylalanine and the hypothetical existence of a dopaoxydase but has led to researches on the oxydases, in general, in relation to pigment formation. Certain authors have supported the view that a polyphenolase plays an important rôle in melanogenesis. Oppenheimer (1926), in a discussion of the present status of the question, concludes that, in the absence of other proof, dopaoxydase may be considered as the constantly present orthodiphenoloxydase, which is identical with the iron-containing chromoöxydase. Others, e.g., Heudorfer (1921), deny the characteristic phenomena of enzyme action in melanogenesis on the significant observation that the dopa reaction and also post-mortem pigmentation can be obtained in sections of boiled skin.

While the development of this stage of the problem was in progress, biochemists were advancing our knowledge of oxidation-reduction phenomena and the important researches of F. Gowland Hopkins on glutathion were published. The sulfhydril bodies are considered as typical models, for the study of hydrogen transfer from one body to another. Being constituents of the cells they act as acceptors and donors, thus regulating the cellular metabolism. Oppenheimer concludes that, in the broader concept of the enzyme theory, glutathion being present in the cell is a ferment "sans phrase", because it is a biological catalyzer.

The reversible sulfhydril system, cystein-cystin, has been studied by Abderhalden and Wertheimer (1922, 1923) and by Mathews and Walker (1909). In the skin, reduced glutathion has been investigated, histochemically, by Walker (1924–1925) and by Kaye (1924) and quantitatively determined, by chemical methods, by Kamiya (1928).

In the morphological studies of the problem, attention has been largely focussed on the rôle played by the nucleus and has led to the consideration of pyrenophil bodies, and plastosomes as a colorless substratum in the evolution of the pigment granule. While many morphological details

have been studied, no consideration has been given to the chemical reactions of the nuclear components. It has been established that nucleoproteins are composed of nucleic acid and basic protamines or histones. Nucleic acid is considered the same in all animal cells and this implies a similarity of function. Mathews (1921) attributes to nucleic acid the function of a supporting structure which aids in maintaining the viscosity of the nucleus. The protamines are considered to play a more important part. According to Kossel (1896, 1906), who has made extensive studies on these basic proteins, protamines are firmly united with the nucleic acid, are located in the nucleus and may be liberated at times.

In view of these newer concepts, the experiments included in this report were undertaken in an attempt to determine the relations of the sulfhydrils in the cytoplasm and the protamines in the nucleus, to the phenomena of pigmentation. This investigation includes chemical and morphological studies. Observations were also made on the phenomena of albinism, post-mortem pigmentation and on the effect of light. The color reaction between protamines and sulfhydrils was found to be reversible. This may be a possible factor in the process of pigment elimination.

Histochemical, qualitative demonstration of reduced glutathion in skins of various colors by the nitro-prusside reaction. Since the presence of reduced glutathion in the living, metabolically active strata of skin has been demonstrated, it is of interest to determine if there is any difference, in this respect, in skins of various colors. Accordingly, observations were made on skin of albino rats and their piebald variety, rabbits and guinea pigs of the following colors, black, gray, red, white (dominant) and albino (recessive whites). Small pieces of fresh skin were imbedded in a small block of 3 per cent agar, frozen and cut 30μ thick. Thinner sections oxidize too rapidly and give a less distinct reaction. The sections were immediately immersed, for five minutes, in a saturated solution of ammonium sulphate and were then placed on a slide. A few drops of freshly prepared 5 per cent solution of sodium nitroprusside was applied and one drop of strong ammonia added. The typical magenta colored band appeared in all epithelial layers. The intensity of color was not uniform and the reaction was very transient. Forty-five seconds was the average time after which it faded and finally disappeared. No difference whatever was detected, either in intensity or duration, in the various skins examined. The reaction was identical in all colored skins as well as in the white and in the skins of albinos. In the piebald rats there was no difference in the reactions obtained in the white and black portions of skin. Sections through borderline regions showed an equal reaction in both pigmented and non-pigmented areas.

Quantitative determination of reduced glutathion in skins of different colors. The same varieties of skins were used, for the chemical determination of

the amount of reduced glutathion, as were employed in the histochemical observations. A weighed amount of shaved and washed (in water) skin was passed through a mincing machine three times or until a pulpy mass was obtained. This was rubbed with quartz sand, in a mortar, and three volumes of 10 per cent trichloracetic acid solution added gradually and allowed to act for 15 minutes. The supernatant liquid was filtered and titrated by Tunnicliffe's (1926) iodometric method, in which N/100 solution of iodine is used. As indicator we employed starch solution according to the Perlzweig and Delrue (1927) modification. The computation was according to Tunnicliffe's equation, $2R.SH + 2I = RSSR + 2HI$, in which 1 cc. N/100 I = 2.5 mgm. of reduced glutathion. The average percentage obtained in skins of rabbits, guinea pigs and rats, was 0.042. There were slight individual variations in every group examined, which might be explained by differences in age and diet. Special attention was given to dominant whites and recessive whites (albino) but no significant difference was found in the results. These observations indicate that there is no difference in the amount of reduced glutathion present in the skins of different animals, regardless of color.

Histochemical demonstration of thymonucleic acid by Feulgen's nucleal reaction. Although nucleic acid has been found to be similar if not identical in nuclei of all animal cells, it was desirable to determine its presence in the nuclei of the stratum germinativum, as a possible factor in pigment formation in skin. The same varieties of skins were used. Voss' method (1925) of staining in bulk with Feulgen's reagent was employed. Pieces of skin, fixed in mercuric chloride, were kept 1 hour in N/1 hydrochloric acid, at room temperature, transferred to fresh N/1 hydrochloric acid and kept 1 hour and 45 minutes at 55°C. They were then placed for 30 minutes in sulfurous acid solution, stained for 4 hours in colorless fuchsin-sulfurous acid and washed with sulfurous acid. Paraffin sections were cut 5μ thick. In general all nuclei appeared intense violet. Of other elements, only elastic fibers were stained. No difference in the reactions was obtained in the skins of different colors although there was some difference in intensity, sometimes in the same section or in different sections of skin from the same animal. Some nuclei fail to give a positive reaction, suggesting that they may contain very little or no nucleic acid. However, from examination of a large number of sections, it was determined that the Feulgen reaction did not demonstrate any significant differences among the various skins studied.

Extraction of protamine from skin. The method employed was that of Kossel (1898), who worked with fish sperm. Mutatis mutandis, the following technique was adapted for skin: the skins were first shaved and washed, separated from subcutaneous tissue by scraping, cut into small strips and extracted for 24 hours with 2 per cent sulfuric acid, in an incu-

bator at 48°C. The resulting detritus mass was rubbed with quartz sand in a mortar and again extracted for 24 hours. The product was strained through gauze to remove the coarser tissue detritus and the liquid was centrifuged. The supernatant liquid was then filtered, in vacuo, through a Seitz filter. A clear, amber-colored, thick, colloidal liquid was obtained which, in the refrigerator, changed to a gelatinous mass. This filtrate was precipitated with three volumes of 95 per cent alcohol. White floccula appeared in 5 to 7 minutes and were allowed to sediment overnight. The supernatant liquid was syphoned off and the precipitate redissolved in warm distilled water, from which it was again precipitated by 95 per cent alcohol. The final precipitate was dried in vacuo over calcium chloride. The resulting dry mass was pulverized. Further precipitation with sodium picrate was omitted. The products thus obtained from the various skins were preserved for further studies.

A 1 per cent solution was prepared from each extract and the following reactions observed. The biuret reaction was positive in all of the solutions; other protein reactions as Milon, xanthoproteic, Adamkiewicz, Hopkins-Cole and Lieberman were negative. The solutions were strongly alkaline to litmus and were digestible by trypsin. With silver nitrate they yielded a black precipitate. These tests showed that, by the extraction, a protamine was obtained.

Reciprocal reaction between protamines and sulfhydrils. Kossel (1894) observed that basic histones were precipitated by acid proteins. Picton and Lindner (1897) have shown that reciprocal flocculation occurs also with hydrophile colloids when the components have antagonistic charges. Hunter (1907) has studied clupein in its combinations with ovalbumen, leim, hemielastin, casein and edestin. Beth af Ugglas (1914) has examined protamine-hemoglobin and protamine-casein reactions. Since only protamine bases react with other proteins he added alkali to the protamine sulphate and thus liberated the base. Wilson (1925), discussing this property of proteins, states,

> Proteins readily combine with other proteins, and many of the proteins of the tissues and tissue-fluids are believed to be thus united, forming complexes which, as a number of biochemists have suggested, may be characteristic of these several tissues and thus offer a key to their physiological individuality. . . . . It is also of cytological interest that some of the natural pigments (e.g., haemoglobin or the phycoerythrin of plants) are conjugated proteins.
>
> A remarkable peculiarity of the proteins, is their *amphoteric* character, i.e., the capacity of one and the same protein to form a salt-like compound with either an acid or a base. This property is due to varying electrical charges, the protein radical being in the one case electro-positive, in the other electro-negative. Some proteins, such as protamine are predominantly basic, others predominantly acid, such as casein, or the "nucleins."

Up to the present, according to all authors, it is not known what the chemical mechanism of such combinations may be, nor is anything known concerning the grouping of atoms in the protein molecule which makes such a reaction possible. It is known, however, that it must be a group easily destroyed by hydrolysis. In view of these theoretical considerations and the axiom that two colloidal substances of antagonistic electrical charges, in proper concentration, act upon each other causing a precipitatation, we were led to examine, experimentally, the reciprocal reaction of reduced glutathion and protamine.

A supply of reduced glutathion was obtained through the courtesy of Prof. Carl Voegtlin, U. S. Department of Agriculture, to whom I am exceedingly grateful. Using the powdered protamine extracts, a series of tubes were prepared containing protamine 1 per cent and reduced glutathion 0.1 per cent. They were buffered with phosphates to pH 7.38. To each tube a crystal of thymol was added to prevent bacterial contamination. Parallel series without thymol were placed in the refrigerator.

After 24 hours the content of all tubes became opalescent and during the next 24 hours all contained a white flocculent precipitate. This precipitate remained white indefinitely. When tubes so prepared were kept without thymol, at room temperature, or if those which had remained white in the refrigerator for as long as six weeks, were returned to room temperature, a change in color appeared in all after two or three days. The precipitate turned gray and soon became black. Microscopic examination showed floccula and numerous bacteria, predominantly a spore bearing bacillus. It appeared that bacterial enzymes, probably of a proteolytic nature, had altered the original white flocculent precipitate. The chemical identity of the precipitate, the identity of the bacteria and the nature of the supposed enzyme were not determined. If allowed to stand longer, the black precipitate becomes lighter in color, first dark gray, then pale gray and finally white. In two to three weeks all precipitates were again white and remained white. This experiment shows that the black color reaction occurs with protamine extracted from black or other colored skins as well as with protamine from white or albino skins. Therefore, it appears that, chemically, protamine is identical in all skins regardless of color. It also is apparent, from these experiments, that the reaction is reversible.

Determination of the optimal pH. Sörensen's phosphate buffer solutions were used. Solutions of protamines (1 per cent) and glutathion (0.1 per cent) were buffered from pH 5.2 to pH 8.04. No preservatives were used and the tubes were kept at room temperature. In the tubes of pH 5.2 to pH 7 no precipitate formed; the liquid remained clear. At pH 7 and above a white precipitate appeared which became black after three to four days. As already mentioned, the color reaction is reversible. In three weeks the black precipitate in all tubes became white and remained so. From

these experiments, which have been repeated many times, it appears that a sulfhydril-protamine precipitate occurs and gradually becomes black in an alkaline medium whose range extends from the neutral point to pH 8.04.

Boiling does not destroy the reaction. Another series of solutions in phosphates of pH 7.38 was prepared and each tube heated to the boiling point, after which the tubes were kept open to permit bacterial contamination. The reaction occurred in the same manner as in the previous experiments. Boiling did not destroy the factors concerned. This experiment was suggested by the known thermostable quality of both reduced glutathion and protamine. The cleavage of glutathion has been discussed in two recent publications.

Grossman, Dyckerhoff and Eibler (1930) examined the spontaneous cleavage of glutathion in aqueous solutions. They employed the carboxylpolypeptidase of the pancreas and split glutathion into glycine and diglutamyl-cystine. Masson (1931) hydrolysed with erepsin and found that the reaction takes place in two stages. First, glutathion is hydrolysed to pyrrolidon carboxylic acid and cysteinil glycine, and secondly cysteinil glycine is further hydrolysed by erepsin. From the three components of reduced glutation, cystein will be considered because it is a sulfhydril body and further because, according to certain authors (Mathews loc. cit.), reduced glutathion behaves in all respects like cystein. There seems to be no change in its properties due to the union with glutamic acid. Oppenheimer (loc. cit.) states that the determining process is the reversible change of the SH group into the SS group whether it is cystein, thioglycolic acid or glutathion, and that the coupling to glutamic acid only serves the purpose of rendering the dehydrated phase water soluble. Otherwise cystin would be excluded from the reaction as an insoluble body. Mathews and Walker (loc. cit.) have studied the oxidation of cystein and found the rate of oxygen absorption at different concentrations of H ions as measured by millimeter negative pressure of Hg to be 4.6 at pH 4; 15.5 at pH 5; 25.7 at pH 6; 35.7 at pH 7; 55.5 at pH 8; 45.2 at pH 9; 40 at pH 10; and 10 at pH 12; Abderhalden and Wertheimer (loc. cit.) published a series of articles on this same problem. They found cystein stable in an acid medium and its optimum of spontaneous oxidation at pH 8. As Mathews remarks, in this susceptibility and position of the optimum, cystein oxidation resembles protoplasmic oxidations.

Cystein protamine reaction. Similar series of tubes as in the previous experiments were prepared. The cystein used was a hydrochloride and the 1 per cent solution was neutralized with NaOH. Protamine (1 per cent) was added to the solution. These solutions were buffered with phosphates to give the complete range from pH 5 to pH 8.04. In all tubes from pH 7 to pH 8.04 a white, flocculent precipitate formed after 8 hours,

which became black by the third day. Below pH 7 the liquid remained clear, no precipitation occurred. This series of experiments permits the interpretation that cystein oxidises in alkaline media and that the resulting cystin, in statu nascendi, reacts with protamine. To verify this, samples were pipetted from the tubes and tested with the nitroprusside reaction. This reaction was positive in samples on the acid side, disappearing rapidly on the alkaline side. Where the black precipitate was present the nitroprusside reaction was negative.

Cystin protamine reaction. Having found the oxidized form of the sulfhydril, namely, the cystin, to be the factor reacting with the protamine the color reaction with pure cystin was investigated. A preparation of l-cystin insoluble in aqueous media was used. When emulsions of cystin and protamine in phosphate buffer solutions, from pH 5.1 to pH 8.04, were allowed to stand for three days or longer a darkening took place. In all tubes above pH 7 the precipitate became gray after three days and black after four. The color appeared at the periphery in the form of a ring and spread toward the center until finally all of the precipitate was black. In some tubes 3 to 4 parallel rings formed, showing Liesegang's zones, characteristic of the precipitation of colloidal substances. The precipitate became white after two to three weeks. During this change some of the rings become brown or reddish brown. The resulting figures resembled the patterns seen on the wings of lepidoptera. From these results and from the insolubility of cystin in aqueous media, it seems probable that by the action of bacterial enzymes further changes may occur, forming products which yield the color reaction.

The catalytic action of iron. The black color reaction between a sulfhydril body and a protamine suggested tests for the presence of iron. With ammonium sulfide, our protamines reacted positively, an olive green color appearing in all solutions. To exclude the possibility of impurities, fresh preparations of protamines were made with precautions to avoid any possible contamination. All solvents and reagents used were tested for iron and found negative. The skins were first washed in distilled water to eliminate hemoglobin. The first pressing was done through sterile gauze. The vacuum filter and the Seitz pads were tested by first filtering a solution of 1 per cent H_2SO_4, which gave a negative iron reaction. The protamines obtained must therefore have contained the iron. Spitzer (1897) has obtained iron from various organs. According to Macallum (1908) organic iron is contained in egg yolk, in the liver, in the nucleoprotein of milk and in the chromatin of nuclei. The doubt of the existence of iron intravitam comes, according to him, from the fact that it is impossible to get a positive reaction with fresh organs. He calls this iron, "masked iron," and shows that reagents such as sulfuric, hydrochloric and nitric acids, in dilute solutions, are able to free it. Concerning the oxidation of

cystein we are led to the discussion of the rôle played by iron and to the opposing views of Warburg and of Abderhalden. Warburg (1925) and Warburg and Sakuma (1923) show that iron-free cystein is not autoxidizable. Harrison (1924) has proved that traces of iron intensify the oxidation of cystein and reduced glutathion. Warburg further considers that all stable oxido-reduction systems of which the sulfhydrils are the most important in the cells can not act if iron is lacking. This fact does not diminish the importance of the organic component. The metal and the organic part are equally important factors. Abderhalden and Wertheimer (loc. cit.) oppose these views and in their experiments have obtained oxidation of absolutely iron-free cystein. As far as our experiments permit conclusions, the formation of the flocculent black precipitate, which in the first place requires the autoxidation of cystein in the presence of an iron-containing protamine, supports Warburg's view.

Histochemical demonstration of iron in the skin. Pieces of colored, white and albino skins were fixed in 95 per cent alcohol for 24 hours, imbedded in paraffin and sections were cut 5μ thick. In order to obtain hydrolysis of nucleoproteins a number of slides were placed, for 24 hours, into 1 per cent sulfuric acid in alcohol (95 per cent). This corresponds to our technique in protamine extraction. It also follows Macallum's idea in freeing the molecularly bound or masked iron. After thoroughly washing out the acid in alcohol, the slides were placed in a mixture of equal parts of 1.5 per cent potassium ferrocyanide and 0.5 per cent hydrochloric acid. A number of non-hydrolyzed sections were placed in the same solution to serve as controls. In the non-hydrolyzed sections the blue iron reaction was absent in the stratum germinativum, nuclei were unstained, while showing slight blue color of the fibrous and elastic fibrils in the cutis and a deep blue sharp staining of the keratohyalin granules. In the sections which had been hydrolyzed the picture was entirely different. At a low magnification the stratum germinativum appeared as a continuous blue band. The keratohyalin granules were unstained and in the cutis the nuclei of all cells were stained blue. Examined with a higher magnification all nuclei in the stratum germinativum were blue, the nuclear membranes very sharp and deep blue and the nuclear substance distributed within the nucleus in irregular coarse blue granules. In some nuclei the blue granules accumulated at the distal pole forming caps. In hair follicles the same figures appeared in the strata which histogenetically derive from the epithelium. A stronger reaction in the basilar columnar cells of the stratum germinativum was observed and a weaker one in the stratum spinosum. Concerning the pre-existing pigment granules we found the following: in regions of weaker pigmentation small, fine, brown granules lay close to the nuclear membrane surrounding, as a ring, the blue nuclear mass. In regions of more intense pigmentation, the pigment granules form the well

known polar caps which in our preparations would fit exactly on a layer of blue nuclear substance. Some granules are partly blue and partly melanin. In order to verify these results we used skin from the soles, anticipating clearer results due to the thickness of both the stratum germinativum and stratum corneum. Identical figures were obtained, as described above, only more conspicuous. The non-hydrolyzed sections look exactly like photographic negatives of the hydrolyzed ones.

Interpretation. In the non-hydrolyzed sections the nucleoprotein is intact and the iron of the nuclei is masked. Only in the stratum granulosum, where splitting of nuclear substance has taken place, is iron freed and appears in the keratohyalin granules. In the hydrolyzed sections the free iron of the keratohyalin granules is dissolved away and the molecularly bound iron of the nuclei is freed by the cleavage of nucleoproteins into protamines and nucleic acid. After demonstrating, in vitro, that skin protamines contain iron it seems possible that the demonstration of iron in the nuclei, after hydrolysis, might prove the granules to be protamine. Although protamines thus far obtained from other organs, e.g., fish sperm, do not contain iron, the skin protamine does contain it. This is possible, since protamines are complex proteins and variations may be due to rearrangements of the component amino acids or through linking of new elements as, in this case, the iron. This may explain, also, why in other organs, where sulfhydrils and protamines come together, pigment does not form. From the presence of the sulfhydrils in the cytoplasm and the iron containing protamine in the nucleus, and further having obtained, in vitro, a black precipitate through their reciprocal interaction we may assume that the evolution of the pigment granule from a colorless plast is an intracellular protamine-cystin linkage. Melanogenesis is nature's micro-color reaction in which the cystein-cystin complex acts as indicator and brings in evidence a phase of nuclear metabolism, namely, the cleavage of protamine and its diffusion into the cytoplasm. The colorless plastes, therefore, may be considered as protamine. In itself the cleavage of protamine as a biochemical process is not necessarily connected with melanogenesis. These facts suggest further investigations concerning the nature of chromidia, plastosomes and basophilic material of gland cells. Our results are in harmony with Meirowsky's theory (1908) which states that the nucleus as such is never destroyed and that only a part of the nuclear substance passes into the cytoplasm. From our experimental results the suggestion may be made that the process of melanogenesis, in vivo, may occur as follows: a stimulus, generally light, activates the intracellular enzymes, as nuclease, nucleotidase, which cleave nucleoprotein (chromatin) into protamine and thymonucleic acid. Protamine passes by osmosis through the nuclear membrane into the cytoplasm and at the neutral point a precipitate occurs forming the colorless plastes. The alkalinity of the medium further makes

the autoxidation of cystein possible. The iron content of protamine acts as a co-ferment. In time the action of cystin on protamine runs its course and the plastes become pigment granules.

Post-mortem pigmentation. The phenomenon of post-mortem pigment formation as shown by Meirowsky (1909) supports the validity of our theory of cystin protamine linkage. Meirowsky assumes that, through autolysis, decomposition products result which are then changed into pigment by a ferment. Heudorfer (loc. cit.), Königstein (1909), Neuburger (1920) and Winternitz (1919) all confirmed Meirowsky's experimental results. Neuburger describes the appearance of new granules through incubation. In our experiments, pieces of colored, white and albino skins of guinea pigs and rats were incubated for 24 hours at 55°C., fixed for 24 hours in 95 per cent alcohol and imbedded in paraffin. Sections were cut 5μ thick and subjected to the ferrocyanide iron reaction. On the assumption that the autolytic ferment had split the nucleoproteins (chromatin), the freed protamines ought to give a positive reaction histochemically. The results obtained were as anticipated. A diffuse iron reaction occurred. Protamine freed from the nuclei seems to permeate the skin throughout. The experiment was repeated with human skin and similar results were obtained. On the other hand, the nitroprusside reaction, applied to frozen sections of incubated skin, was negative, indicating oxidation of the reduced sulfhydril. Once freed the protamine reacts with cystin in the usual way. Our experiments, in vitro and histochemically, confirmed what Meirowsky had postulated theoretically. Further confirmation of our cystin-protamine theory is afforded by an experiment by Neuburger (loc. cit.). He observed that pieces of skin in oxygen-free medium remained unchanged even at 58°C. while those in a rich oxygen medium showed increased pigmentation. He considers the process an oxidation. We confirmed this idea by an experiment, in vitro. In a series of tubes containing cystein-protamine in phosphates, buffered from pH 5 to pH 8, one drop of 1 per cent solution of potassium cyanide was added to each cubic centimeter. The black color reaction did not occur. Oxidation had been inhibited and the nitroprusside reaction remained positive. This demonstrates that autoxidation of cystein is the conditio sine qua non in melanogenesis. The cystein-protamine theory also explains Heudorfer's experiment in which he obtained post-mortem pigmentation when incubating boiled skin. We have already shown that the chemical reaction occurs after boiling the solutions. Both factors, cystin and protamine are known to be thermostable. As to the presence of increased granules in incubated skin, our results differed from those of other authors. Heudorfer (loc. cit.) found them more numerous in pigmented cells and less in others, using a 0.1 per cent silver nitrate solution. In our experiments, paraffin sections of incubated skins were placed in a 1 per cent solution of silver

nitrate, in daylight, and after 24 hours reduced with 2 per cent acetic acid. The granules were increased in pigmented skins and no granules at all were present in white and albino skins, although the iron reaction was positive in all. This demonstrates that in colored skin the diffusing protamine precipitates, yielding the colorless plastes, while in white and albino skin it does not, hence the absence of black silver protein granules. This is in harmony with our chemical experiments. Protamine precipitates other proteins in an alkaline medium only. From the literature it is found that all previous incubation experiments have been performed with human skin. White human skins contain a little pigment and there is lacking a condition similar to that in the skin of a white or albino animal. This may explain our different findings.

Influence of light and ultraviolet rays on the chemical reaction. Aberhalden and Wertheimer (loc. cit.) and also Gerwe (1931) have studied the influence of ultraviolet rays on the autoxidation of cystein and found it negative. Our protamine-cystein reactions have shown that the first step in the formation of the black precipitate was the autoxidation of cystein, as proved by the disappearance of the nitroprusside reaction. In order to investigate, in relation to our problem, the significance of the negative results of the above mentioned authors, we prepared series of tubes as previously described, containing protamine-glutathion and protamine-cystein solutions, buffered from pH 5 to pH 8. These were placed in an absolutely dark room. The reaction, i.e., the appearance of the black floeculent precipitate, occurred in the same manner as in the tubes kept in daylight. For observations on the influence of ultraviolet rays, we prepared solutions of protamine and reduced glutathion and of protamine and cystein in phosphates buffered at pH 7.38, placed them in open porcelain cups and subjected them to the rays of a quartz mercury lamp for one hour at 30 cm. distance. These solutions were then transferred to test tubes and kept as described in the other experiments. The reaction ran the same course as in non-rayed, control solutions. From these experiments, it is demonstrated that light has no influence on the dynamics of the chemical reaction, sensu strictiori, although it influences the biological factors operating in vivo.

Influence of ultraviolet rays on melanogenesis in vivo. All authors who have studied the action of ultraviolet rays on skin and pigmentation have reported an increase in the number of pigment granules and also of colorless pre-pigment plastes. Lutz (1917) could always obtain a more pronounced dopa reaction after raying and also a stronger silver nitrate blackening. We were led to investigate, in rayed skin, first the sulfhydril reaction, second the iron reaction as an index of protamine cleavage and third the silver reaction as an index of protamine precipitation. We used guinea pigs, albino, black and white, and pure black; rats, albino and the

black and white variety. The members of one group were rayed only once, for 30 minutes at 25 cm. distance and were sacrificed after 3 hours. Another group was subjected to the rays for 30 minutes at 25 cm. distance, three times in three consecutive days, sacrificing the animals 24 hours after the last raying. Frozen sections of these rayed skins always showed a positive nitroprusside reaction, which indicates that the sulfhydrils are still in the reduced form. Pieces of the skins were fixed in alcohol and imbedded in paraffin. The iron reaction, in the cells of the stratum germinativum of the skin rayed only once, was negative in guinea pigs and weakly positive in rats. This might be due to thinner skin of rats. When sections of these 30 minute rayed skins were hydrolysed with 2 per cent sulfuric acid for 24 hours and then subjected to the iron reaction they all reacted strongly positive; all nuclei were blue. This indicates that 30 minutes' raying did not split the nucleo-proteins in guinea pigs' skin and just started it in rats. In the skins rayed three times, the iron reaction was positive in all. In blacks we obtained, in the basilar cells of the stratum germinativum, a stronger reaction in the nuclei. Some of the melanin granules were partly blue and partly brown. In albino and in white skin the nuclei were blue but granules were not detected in the cytoplasm. For further confirmation of these facts the silver nitrate method was employed. Paraffin sections of the skins rayed three times were placed in a 1 per cent solution of silver nitrate for 24 hours in daylight, reduced in 2 per cent acetic acid, washed in water and alcohols and mounted in Canada balsam. In black skins an increase of granules, which was better seen in tangential sections, occurred. In places they formed continuous black bands. No granules were found in albino and white skins. In sections of black and white skins, through the borderline of a pigmented area, presence of the granules abruptly ends at the natural line of pigment demarcation. The interpretation of these results may be that the ultraviolet rays influence the nucleoprotein metabolism by activating such enzymes as nucleases and nucleotidases and thus splitting of protamine occurs. The rest of the reaction follows the course already discussed. The increased number of plastes and granules in colored skins is favored by the alkalinity of the cytoplasm which is essential for the precipitation of protamine. The absence of granules in white and albino skins is evidence of acidity of the cytoplasm. In our experiments with extracts we found that precipitation does not occur in an acid medium, and confirmed this fact in our incubation experiment. The dopa reaction is stronger in rayed skin, as Lutz found, and as we also observed. This may be considered as an index of increased alkalinity due to protamine diffusion. The increased number of granules which react positively with dopa are the granules which can also be stained with the iron reagents and are therefore protamine. In vivo, hyperemia is the first physiological response to the rays and is the factor initiating all

the succeeding chemical processes, as enzyme activity and the cleavage of protamine and sulfhydrils. The increased pigmentation through light occurs also post mortem, as in the experiment of Lignac (1923). The rays, therefore, activate the autolytic ferments as well.

The dendrite cells. Without entering into a discussion of the nature of these cells and of the physiological functions attributed to them, it is of interest to communicate here an observation made in the course of our experiments with ultraviolet rays. In tangential sections of rayed skins of black or other colored animals these cells form a continuous net of brown-black anastomoses. In white and albino skins they are absent. In sections through border lines of black and white areas the presence of dendrite cells ends abruptly at the borderline of natural pigmentation. It is obvious, therefore, that the dendrite cells are present only in areas where the basilar cells contain pigment.

The pH in the skin. Przibram and Brecher (1919) found that albino skins have a stronger acidity than the colored skins. Their method was a titration of watery skin extracts with a N/1000 solution of sodium hydroxide, using phenolphthalein as indicator. They also deduct, that the gray hairs are due to a general systemic acidity. Other methods have been employed by other authors. Sharlitt and Scheer (1923) and Sharlitt and Highman (1923) have applied colorimetric methods on the living skin using bromphenol blue, bromcresol purple and methyl violet as indicators. They determined pH 5.5 as the average for the skin. Memmesheimer (1924), using similar methods, obtained values between pH 5.2 and pH 5.8. The same indicators have been used in injections producing a vital staining. Rous (1926) studied the color reaction of skin grafts by injecting phenol red into the peritoneal cavity. Micro-surgical methods have been applied by Schmidtmann (1924), namely, by introducing into the cells a small crystal of an indicator. The values obtained were pH 6.8–6.9 in the stratum basilare, pH 7.3–7.5 in the stratum spinosum and pH 6.0 in the stratum corneum.

Bloch and his associates found the dopa reaction always negative in albino and white skins. His opponents pointed to the spontaneous oxidation of dopa in an alkaline medium and considered this reagent only as an indicator. To test this question, we prepared a series of tubes containing a 1 per cent protamine solution buffered in phosphates from pH 5.58 to pH 8.04. To each tube were added 3 drops of a 0.1 per cent dopa solution. After 24 hours the tubes pH 5.58 and pH 5.90 remained colorless; pH 6.23 became pale gray, pH 6.81 and pH 6.97 dark gray and pH 7.16, pH 7.38, pH 7.73 and pH 8.04 black. A black precipitate appeared in all tubes over pH 7. This shows that, although not a sharp and precise color indicator, dopa is reliable enough to demonstrate variations in the pH conditions of the media. The experiments of Bloch, therefore, have indicated

that the cells of the stratum germinativum in white skins have a higher acidity, which according to our experiments must be below pH 5.58. Bloch's diffuse gray reaction is a coloring of the cytoplasm and his granular type of reaction is the coloring of the protamine granules. A similar experiment was performed with adrenalin. To avoid the influence of added substances in commercial adrenalin hydrochloride solutions, a synthetic epinephrine base preparation was used. The acid solutions below pH 6.23 remained colorless, the others changing gradually to brown, becoming dark brown at pH 7.73 and pH 8.04. Therefore, epinephrine, like dopa, should be considered only as an indicator of the pH. The brown granules obtained by some authors are probably protamine stained with adrenalin.

The silver nitrate reaction may also be used as an indicator of alkalinity or acidity. Miescher (1922) found that an alkaline reaction is necessary for the formation of black silver melanin and that acidifying prevents it. We prepared solutions of protamine (1 per cent) in N/10 NaOH, N/20 NaOH and N/10 HCl. To each tube was added 1 drop of 1 per cent $AgNO_3$ per cubic centimeter of the solution. After 24 hours the first series (in N/10 NaOH) were medium brown, the second series (in N/20 NaOH) red, resembling the color of fresh muscle, the third (in N/10 HCl) opalescent. In another series of tubes containing 1 per cent solution of protamine, buffered with phosphates from pH 5 to pH 8, 1 drop of 1 per cent $AgNO_3$ was added to each cubic centimeter. In the alkaline solutions a fine granular black precipitate developed, and in the acid tubes a reddish brown liquid with very little precipitation. Histochemically, the reaction runs a parallel course. In sections of skin of normal animals, the silver nitrate reaction is strongly positive in the colored variety and negative in albinos and whites. In sections through borderline of black and white areas the reaction ends abruptly at the line of demarcation of natural pigment. In incubated skin, silver granules were obtained only in colored skins, never in whites and albinos, which indicates absence of precipitation and this, as we have already shown, is due to acidity of the medium (cytoplasm). In ultraviolet rayed skins the same phenomenon occurred. In tangential sections, especially, the silver precipitate forms continuous black bands.

Albinism. In view of the acidity of the stratum germinativum in albino skins, as demonstrated by the dopa and the adrenalin reactions, albinism could be explained on the following hypothesis: the cells of the stratum germinativum in albino skin have a pH below 5.58. The diffusing protamine can not raise the pH above the neutral point. The medium being acid the sulfhydril is in a stable condition and does not undergo autoxidation. This further prevents the formation of the cystin-protamine complex which becomes the black precipitate.

In relation to certain problems in genetics, our results indicate the hypothetical nature of the Bateson presence-absence theory. We find both

sulfhydril and protamine present in colored and in albino skins. The difference in the reaction is in the state of acidity or alkalinity of the medium. This does not imply two antagonistic allelomorph characteristics, but merely a gradual quantitative variation of a single factor. The phaenotypes albino, white and colored are genotypically identical so far as protamine and sulfhydrils are concerned. The problem concerns the variations of the third factor and the levels of variation at which the pH ceases to be dominant and becomes recessive.

Elimination of pigment. Miescher (loc. cit.) describes the elimination of pigment from the epidermis through the chromatophores, small superficial connective tissue cells located in the papillary layer. Through some damaging factor pigment is set free from the epithelial cells and is taken up by the chromatophores. The cutis is only an intermediary station in the transportation process. The fact that pigment can be traced from epidermis to lymph glands without increasing the chromatophores speaks against Miescher's hypothesis. According to other authors, the transportation of pigment occurs through the lymphatic vessels. Another factor to be considered is the size of the pigment granule. It is small in the epidermis and coarse in the chromatophores. Miescher explains this by supposing that the pigment leaves the epidermis in a dissolved state, is reprecipitated in the chromatophores and probably again dissolved and reprecipitated in the lymphatic nodules, where it reappears as fine granules like in the epidermis. This solubility of the pigment granule seems hypothetical to Meirowsky (loc. cit.). This solubility in the tissue extract has not been demonstrated. Voinov and Voinov (1924) in their study on Simulum larvae found that toward the end of the larval stage the pigment granules are dissolved without a residuum. Verne (1920) in his studies on decapodes found that in the depigmentation process there remains no substratum. Linking these morphological facts with what we have already learned on the chemical side of the problem attention may be directed to the work of Beth af Ugglas (loc. cit.). In studies on protein-proteinates this investigator found that the precipitate of a protein into another protein is redissolved when one of them, namely, the acid one is in excess. The reversibility of the color reaction as demonstrated in our experiments may explain the elimination of melanin in form of colorless succedaneous products. What the chemical changes are which transform the black precipitate into white is, at present, unknown. It may be a change in pH toward the acid side or perhaps a progressive oxidation. The protamines thus far known contain, in varying percentages, the following amino acids: arginine, lysine, histidine, tyrosine, alanine, valine, serine, proline, tryptophane. They differ from other proteins in the fact that through cleavage they yield mostly basic diamino acids and very little monoamino acids. Kossel (loc. cit.) found that in protamines there are

always two molecules of a basic amino acid, as arginine, lysine or histidine, to each molecule of a monoamino acid. They are, in fact, characterized by high content of bases. A protamine seldom contains all three bases. Protamine solutions react alkaline. In order to elucidate the structure of protamines, Waldschmidt-Leitz et al. (1931) have studied the action of a specific enzyme, the protaminase prepared from pancreas. Its action results in the removal of the basic amino acids such as arginine from the carboxyl radical of the protein molecule. As to the rôle of such basic amino acids as arginine, Guggenheimer (1920) is of the opinion that since the biological function of arginine in the intracellular metabolism is not known, we can only assume that through its basic groups it is best fitted to change into other nitrogenous complexes, amino acids or purin bodies. Further, that in cleaving from the protein complex, it changes the OH ion concentration considerably thus influencing the depending fermentative reactions. The basic amino acids and especially arginine, which is contained in protamines to such a high percentage (80 per cent), may play the predominant rôle in melanogenesis. Further studies of the cystein-cystin system, in its reactions with the basic amino acids, arginine, lysine and the imino acid histidine, are desirable. According to the researches of Emil Fischer and his pupils, the amino acids may be linked together as they are, or probably one or both may first undergo polymerization before linkage. In respect to cystin this has been shown by Fischer and Suzuki (1904).

SUMMARY

The results of experiments reported in this communication support the view that melanogenesis is an intracellular process and a physiological function of the pigment cell. It results from the reaction of cystin on protamine and is therefore limited in the skin to the epithelial layer, the only one containing sulfhydrils. Melanogenesis is nature's micro-color reaction in which the cystein-cystin complex acts as an indicator and evidences a phase of nuclear metabolism, viz., the cleavage of protamine and its diffusion into the cytoplasm. Cystein is present in the cytoplasm within the more complex body, the tripeptid glutathion, and protamine is present in the nucleoprotein (chromatin). Through cleavage due to cell enzymes these two bodies are freed and brought in contact. In vitro a black precipitate is formed when protamine and cystin are brought together in a proper medium. It starts at the neutral point and reaches the optimum between pH 7.38 and pH 8.04. The reaction is slow depending on the gradual autoxidation of cystein. In an oxygen free medium the reaction does not occur. The reaction takes place in the presence of iron. Skin protamine contains iron. The color reaction is reversible. The black flocculent precipitate becomes white in time. The reversibility of the reaction suggests that elimination of melanin probably occurs in the form

of colorless succedaneous products. The derivation of melanin from a sulfhydril body explains the presence of sulfur. Post-mortem pigmentation is related to autolytic changes which produce protamine from nucleoproteins. Ultra-violet rays do not influence the chemical protamine-cystin reaction. They only activate the physiological factors coöperating in vivo towards the freeing of protamine. Our experiments suggest that albinism is related to acidity, which prevents the precipitation of protamine and the autoxidation of cystein. Our results may have a bearing upon pigmentation in Addison's disease, in the light of reported changes in sulfur metabolism in this disease.

I wish to express my gratitude to Prof. J. M. Rogoff for his guidance, for the facilities offered in his department and for helpful criticism in the preparation of the manuscript.

BIBLIOGRAPHY

ABDERHALDEN, E. AND E. WERTHEIMER. 1922. Pflüger's Arch., cxcvii, 131.
1923. Pflüger's Arch., cxcviii, 122.
FISCHER, E. UND U. SUZUKI. 1904. Ber. d. Deutsch. Chem. Gesellsch., iv, 4575.
GERWE, E. G. 1931. Journ. Biol. Chem., xci, 57.
GROSSMANN, W., H. DYCKERHOFF UND H. EIBELE. 1930. Zeitschr. f. physiol. Chem., clxxxix, 112.
GUGGENHEIM, M. 1920. Die Biogenen Amine. Berlin.
HARRISON, D. C. 1924. Biochem. Journ., xviii, 1009.
HEUDORFER, K. 1921. Arch. f. Dermat. u. Syph., Berl., cxxxiv, Orig., 339.
HUNTER, A. 1907. Zeitschr. f. physiol. Chem., liii, 526.
KAMIYA, T. 1928. Nagoya Journ. Med. Sci., iii, 25.
KAYE, M. 1924. Biochem. Journ., xviii, 1289.
KÖNIGSTEIN, H. 1909. Münch. med. Wochenschr., Nr. 45, 2305.
KOSSEL, A. 1894. Deutsch. med. Wochenschr., Nr. 7, 147.
1896. Zeitschr. f. physiol. Chem., xxii, 176.
1898. Zeitschr. f. physiol. Chem., xxv, 165.
1906. Zeitschr. f. physiol. Chem., xlix, 301.
LIGNAC, G. O. E. 1923. Virchow's Arch., ccxl, 383.
LUTZ, W. 1917. Arch. f. Dermat. u. Syph., cxxiv, 233.
MACALLUM, A. B. 1908. Ergebn. d. Physiol., vii, 552.
MATHEWS, A. P. 1921. Physiological chemistry. 3rd ed. New York.
MATHEWS, A. P. AND S. WALKER. 1909. Journ. Biol. Chem., vi, 21.
MASSON, H. L. 1931. Journ. Biol. Chem., xc, 25.
MEIROWSKY, E. 1908. Über den Ursprung des melanotischen Pigmentes der Haut und des Auges. Leipzig.
1909. Ziegler's Beitr., xx, 301.
MEMMESHEIMER, A. 1924. Klin. Wochenschr., iii, Nr. 46, 2102.
MIESCHER, G. 1922. Arch. f. Dermat. u. Syph., cxxxix, 313.
NEUBÜRGER, K. 1920. München. med. Wochenschr., lxvii, 741.
OPPENHEIMER, C. 1926. Die Fermente. 5th ed. Leipzig.
PERLZWEIG, W. A. AND G. DELRUE. 1927. Biochem. Journ., xxi, 1416.
PICTON, H. AND S. E. LINDNER. 1897. Journ. Chem. Soc., lxxi, 568.

PRZIBRAM, H. UND L. BRECHER. 1919. Arch. f. Entw. Mechanik d. Organismen, xlv, 83.
ROUS, P. 1926. Journ. Exp. Med., xliv, 815.
SCHMIDTMANN, M. 1924. Biochem. Zeitschr., cl, 253.
SHARLIT, H. AND W. J. HIGHMAN. 1923. Arch. Dermat. and Syph., viii, 515.
SHARLIT, H. UND N. SCHEER. 1923. Arch. Dermat. and Syph., vii, N. S., 592.
SPITZER, W. 1897. Pflüger's Arch., lxvii, 615.
TUNNICLIFFE, H. E. 1926. Biol. Rev. and Biol. Proc., Cambridge Phil. Soc., ii, 80.
AF UGGLAS, B. 1914. Biochem. Zeitschr., lxi, 469.
VERNE, J. 1920. C. R. de la Soc. de biol., lxxxiii, 760.
VOINOV, D. ET V. VOINOV. 1924. C. R. de la Soc. de biol., xc, 722.
VOSS, H. 1925. Verhandl. de anat. Gesellsch., Jena, xxxiv, 118.
WALDSCHMIDT-LEITZ, E., F. ZIEGLER, A. SCHÄFFNER UND L. WEIL. 1931. Hoppe-Seyler's Zeitschr., cxcvii, 219.
WALKER, E. 1924–25. Journ. Physiol., lix, xxxii (proc.).
WARBURG, O. 1925. Ber. Chem. Gesellsch., lviii, 1001.
WARBURG, O. AND S. SAKUMA. 1923. Pflüger's Arch., cc, 203.
WILSON, E. B. 1925. The cell in development and heredity. 3rd ed., New York.
WINTERNITZ, R. 1919. Arch. f. Dermatol. u. Syph., cxxvi, 252.

ON THE CHEMICAL COMPOSITION OF LYMPH FROM SUBCUTANEOUS VESSELS[1]

J. WILLIAM HEIM

From the Department of Physiology, Harvard School of Public Health, Boston, Mass.

Received for publication October 17, 1932

Information concerning the chemical composition of lymph is extremely scarce and methods of analysis prior to 1925 are subject to so much criticism as to exclude the meagre data afforded by the literature. Meyer-Bisch and Gunther (1) and Petersen and Hughes (2) in 1925 and Arnold and Mendel (3) in 1927 have made fairly complete chemical examinations of thoracic duct lymph from the dog. This lymph is easy to collect. In the main it originates from the highly permeable capillaries of the liver and intestine with slight additions from lymphatics in other abdominal organs and from the limbs. One cannot relate the composition of thoracic duct lymph to lymph from any reasonably limited tissue area.

During the past three years workers in this laboratory have revived the methods of obtaining lymph from subcutaneous vessels which were in daily use in Ludwig's laboratory and have found it easy to obtain sufficient lymph for chemical analysis.

The composition of lymph must have a close relation to the composition of the fluid bathing the tissue cells in the region from which it flows. It is perhaps the true *milieu* in which the physiological reactions of the body take place. Its composition and that of blood taken at the same time are therefore of prime interest in physiology and biochemistry.

TECHNIQUE. Dogs weighing in excess of 20 kilograms were anesthetized with nembutal given intraperitoneally. In unanesthetized animals from which subcutaneous lymph was being collected as described by White, Field and Drinker (4) it has been shown that this anesthetic does not affect the rate of flow or protein content of lymph if appropriate samples are compared, samples taken under similar conditions before and after giving the anesthetic.

Sampling. The sampling of the lymph was as follows. The cervical lymphatic trunks were exposed and cannulated. The lymph was transferred by means of a small pipette to a 15 cc. centrifuge tube containing sufficient heparin to prevent coagulation. Light but continuous massage

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Harvard University.

was necessary to induce a steady flow of lymph. As a rule cervical lymph will not flow with sufficient rapidity to prevent coagulation in the cannula and massage is therefore necessary to obtain the samples. There is a wide variation in the amount of lymph secured from different dogs. Some have very small lymphatics and are "dry," so to speak, that is no amount of massage will induce an appreciable flow of lymph. From other animals the lymph flow is so rapid that it is sometimes possible to collect from 10 to 12 cc. in an hour. Rapid lymph flow could be produced by artificial means such as sugar or saline injections but as this is a study of normal lymph these methods could not be adopted. Before the analyses the lymph was centrifuged with the tubes covered to remove minute clots. A blood sample for comparison with the cervical lymph was withdrawn by means of a syringe from the right jugular vein and immediately centrifuged under oil. This sample was collected in the middle of the lymph collecting period.

METHODS. The following methods were selected after a careful study of the best available. The *protein* determination was made by Kjeldahl analysis of the proteins precipitated by tungstic acid. The digestion mixture was diluted, filtered quantitatively into a volumetric flask and made up to volume. An aliquot portion of this was distilled and titrated in the usual manner. The *non protein nitrogen* determination was made on a portion of the tungstic acid filtrate as described by Folin (5). The *urea* content of plasma and lymph was determined by the method of Folin and Svedberg (6) using the tungstic acid filtrate. In as much as the lymph protein content is approximately half that of plasma only one-half as much tungstic acid as the amount used for plasma is necessary. It was found that if a larger amount of the precipitant was used the filtrate became sufficiently acid to inactivate the urease. The *creatinine* determination was made by the method of Folin (5). The *chloride* content of plasma and lymph was determined iodimetrically by the method of McLean and Van Slyke (7) with the following modification of Lamb and Fairhall (8). The potassium iodide was added to within a short distance of the end point and the resulting silver iodide filtered off. This procedure made it possible to complete the titration in a comparatively clear solution which greatly sharpened the end point. The *sugar* determination was made according to the method of Folin (14). The *phosphorus* determination was made by the Fiske-Subbarow method (9). For the total phosphorus analysis, the organic matter in plasma and lymph was removed by dry ashing in platinum. *Calcium* was determined by the Fiske-Logan method (10). The organic material in the samples was removed by dry ashing in platinum. The *uric acid* analysis was conducted according to the method of Folin (11). The *amino acid* determination was made by the improved Folin-Danielson method (unpublished).

RESULTS. Average values of the determinations of the various constitu-

ents of blood plasma and lymph of the dog are shown in table 1. All quantities except for protein are expressed in milligrams per 100 cc. The protein value is expressed in grams per 100 cc. (per cent value). The R value expresses the ratio of the lymph value to the corresponding plasma value.

Protein. The average lymph protein value was found to be 3.32 with a range of 1.38 to 4.57 in 16 determinations. The fact that cervical lymph contains a considerable amount of protein has been previously observed and indicates that the peripheral capillaries are normally permeable to protein. The fact that the lymph protein values are so high will be viewed with considerable surprise by many interested in this phase of the problem.

TABLE 1

Comparison of the concentrations of some of the constituents in peripheral (cervical) lymph and blood plasma of the dog under normal conditions

	PROTEIN (KJELDAHL)	N. P. N.	UREA	URIC ACID	CREATININE	SUGAR	AMINO ACIDS	CHLORIDES AS NaCl	PHOSPHORUS		CALCIUM
									Total	Inorg.	
	per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
Plasma:											
Average	6.18	32.6	21.7	Trace	1.37	123.0	4.90	678	22.0	5.6	11.70
Range	(5.54–7.23)	(21.1–46.0)	(17.9–28.0)		(1.22–1.54)	(112.0–143.0)		(649–721)	(18.3–26.1)	(4.4–6.9)	(10.85–12.95)
Lymph:											
Average	3.32	34.8	23.5	Trace	1.40	132.2	4.84	711	11.8	5.9	9.84
Range	(1.38–4.57)	(19.8–45.4)	(19.8–33.0)		(1.28–1.49)	(107.0–144.0)		(690–730)	(10.2–13.7)	(4.7–7.3)	(8.93–10.84)
R Lymph/Plasma	0.54	1.07	1.07		1.03	1.08	0.99	1.05	0.54	1.05	0.84
Number of animals	(16)	(10)	(7)	(3)	(7)	(16)	(1)	(7)	(6)	(3)	(11)

Nembutal anesthesia.

Average values for cervical lymph protein in the literature are in the neighborhood of 2.5 per cent while these results are approximately 50 per cent higher. Most of the previous values have been obtained however by refractometric methods which are admittedly in error. They have however been used to indicate the fact that blood capillaries are permeable to protein—not impermeable as has been held in the past. The fact that refractometric quantitations average lower than these made by straightforward Kjeldahl technique makes it if anything a stronger case for the contention that normal capillaries all over the body leak protein.

The average lymph *non protein nitrogen* value was found to be 34.8 with a range of 19.8 to 45.4 in ten determinations. The average lymph *urea* value is 23.5 with a range of 19.8 to 33.0 in 7 determinations. In as much

as the end product of purine metabolism in the dog (with the exception of the Dalmatian) is allantoin, one would not expect to find *uric acid* in plasma or lymph. There is a trace of this compound in both of these fluids however. In their study of the uric acid problem Folin, Berglund and Derick (12) concluded that except in the kidney there is no extravascular uric acid and that the circulating blood is the site of uric acid destruction. Christman and Eckstein (13) in order to investigate this theory made

TABLE 2

A comparison of the concentrations of some of the constituents of thoracic duct lymph with those for cervical lymph

	PROTEIN	N.P.N.	SUGAR	CHLORIDES AS NaCl	CALCIUM	PHOSPHORUS		AMINO ACIDS	INVESTIGATOR
						Total	Inorg.		
Thoracic duct lymph									
	per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	
Serum	7.46		266	602					Meyer-Bisch and Günther, 1925*
Lymph	4.14		283	650	11.7	18.4			
Lymph	4.75		114	659	11.8		2.63	2.4	Petersen and Hughes, 1925**
Serum	5.63	27.2	123	646	10.4		4.3		Arnold and Mendel, 1927†
Lymph	3.56	27.0	124	681	9.2		3.6		
Cervical lymph									
Plasma	6.18	32.6	123	678	11.7	22.0	5.62	4.90	Heim, 1932‡
Lymph	3.32	34.8	132	711	9.8	11.8	5.90	4.84	

* Morphine and ether anesthesia.

** Local anesthesia.

† Amytal anesthesia intraperitoneally.

‡ Nembutal anesthesia intraperitoneally.

intravenous injections of a soluble urate and were able to recover uric acid in thoracic duct lymph. It would be very interesting to analyse human lymph in order to determine whether extravascular uric acid exists normally in animals where it is the end product of purine metabolism. So far experiments in this laboratory to obtain normal human lymph in quantities sufficient for chemical analysis have not been successful.

The average lymph *creatinine* value was found to be 1.40 with a range of 1.28 to 1.49 in 7 determinations. One total creatinine determination was made resulting in a value of 3.90 for lymph and 3.92 for plasma. The average of 16 determinations of the lymph *sugar* content is 132.2 with a

range of 107.0 to 144.0. The average lymph *chloride* value was found to be 711 with a range of 690 to 730 in 7 determinations. The higher concentrations of chlorides in lymph with respect to plasma, the R value being 1.05, may be interpreted in terms of the Donnan equilibrium due to the higher protein content of plasma. The concentration of lymph *calcium* is lower than that of plasma, the R value being 0.84. This is due to the lower protein concentration of lymph with respect to plasma. A part of the plasma calcium is probably loosely bound with the plasma proteins in the form of calcium proteinate thus preventing this fraction of the calcium from diffusing into the lymphatics. Some of this bound calcium does however enter the lymph stream with the protein. The average lymph calcium value is 9.84 with a range of 8.93 to 10.84 in 11 analyses. The average *total phosphorus* content of lymph was found to be 11.8 with a range of 10.2 to 13.7 in 6 determinations. The higher protein content of plasma results in its higher total phosphorus value with respect to lymph, the average R value being 0.54. The average of three determinations of the *inorganic phosphorus* content is 5.90 with a range of 4.73 to 7.35. Almost identical concentrations of *amino acids* were found to exist in plasma and lymph, the value for plasma being 4.90 and that for lymph 4.84. However in as much as only one determination was made these figures cannot be accepted as representative.

Discussion. A survey of the average values of the constituents investigated (table 1) reveals the following facts. Except for protein and substances combined with protein (calcium and total phosphorus) the concentrations of the constituents investigated are nearly the same in plasma as in lymph. Concentrations of these constituents are however invariably higher in lymph than in plasma. The higher lymph concentrations may be due to a number of factors. For example in the case of chlorides, Donnan equilibrium relationships must be considered. According to this theory, the higher concentration of the protein ion in the plasma causes an unequal distribution of the diffusible chloride ions with the result that their concentration is higher in lymph than in plasma. Another factor of considerable importance is the possible concentration of tissue fluid and lymph due to reabsorption of water into the blood stream. Conditions of complete quiescence such as occurred in these experiments with the animal under nembutal anesthesia are very favorable for reabsorption. During activity capillary pressure rises with the result that reabsorption is diminished. These phenomena are strikingly illustrated in the walking dog experiments of White, Field and Drinker (4) in which foot lymph protein increased rapidly during rest indicating a rapid absorption of water.

Table 2 shows a comparison of the concentrations of some of the constituents of thoracic duct lymph with those of cervical lymph.

SUMMARY

Values for the concentrations of protein, non protein nitrogen, urea, uric acid, creatinine, sugar, amino acids, chlorides, calcium and phosphorus in subcutaneous (cervical) lymph of the dog under normal conditions (nembutal anesthesia) together with corresponding values for blood plasma taken at the same time are presented.

The author wishes to thank Dr. Lawrence T. Fairhall for his ever available chemical help and advice.

BIBLIOGRAPHY

(1) Meyer-Bisch, R. and F. Günther. Pflüger's Arch., 1925, ccix, 81.
(2) Petersen, W. H. and T. P. Hughes. Journ. Biol. Chem., 1925, lxvi, 229.
(3) Arnold, R. M. and L. B. Mendel. Journ. Biol. Chem., 1927, lxxii, 189.
(4) White, J. C., M. E. Field and C. K. Drinker. This Journal, 1933, ciii, 34.
(5) Folin, O. and H. Wu. Journ. Biol. Chem., 1919, xxxviii, 81.
(6) Folin, O. and A. Svedberg. Journ. Biol. Chem., 1930, lxxxviii, 77.
(7) McLean, F. C. and D. D. Van Slyke. Journ. Biol. Chem., 1915, xxi, 361.
(8) Lamb, A. B. and L. T. Fairhall. Journ. Amer. Chem. Soc., 1923, xlv, 390.
(9) Fiske, C. H. and Y. Subbarow. Journ. Biol. Chem., 1925, lxvi, 375.
(10) Fiske, C. H. and M. A. Logan. Journ. Biol. Chem., 1931, xciii, 211.
(11) Folin, O. Journ. Biol. Chem., 1930, lxxxvi, 179.
(12) Folin, O., H. Berglund and C. Derick. Journ. Biol. Chem., 1924, lx, 361.
(13) Christman, A. A. and H. C. Eckstein. Journ. Biol. Chem., 1927, lxxv, 201.
(14) Folin, O. and H. Inu. Jour. Biol. Chem., 1920, xli, 367.

AN EXPERIMENTALLY PRODUCED PREMATURE SYSTOLIC ARHYTHMIA (PULSUS BIGEMINUS) IN RABBITS

V. Factors that Affect its Onset, Duration, Arrest and Alteration

WILLIAM F. ALLEN

From the Department of Anatomy of the University of Oregon Medical School, Portland

Received for publication October 26, 1932

This paper is intended to be a study on the afferent control of the heart from the standpoint of trigeminal stimulation as well as an investigation of certain factors which may influence the onset, duration, arrest or alteration of the ventricular premature systoles obtained from insufflation of benzol into the nostrils (trigemino-sympathetic impulses).

At the outset it is important to recognize two factors which have to do with the onset of successive arhythmias, namely, a summation effect and a period of rapidly increasing inhibition: hence it is essential to take a control insufflation graph for each test record taken. Since Heidenhein, Hering, Levy, Kisch and others have obtained a bigeminal pulse from a pronounced rise in blood pressure following occlusion of the aorta, one should always know whether the arhythmia resulting from insufflations was primarily of trigemino-sympathetic origin or whether it was produced largely from the hypertension that usually follows an insufflation. This can be ascertained by taking frequent records of similar rises in blood pressure obtained by mechanical means.

Effect of various agencies on the onset and duration of the bigeminal arhythmia from insufflations. *Rise in arterial pressure.* The procedure used to determine if the rise in blood pressure resulting from insufflation was favorable for an earlier onset of the ensuing arhythmia consisted of attaching an equalizer to the abdominal aorta, which when functioning would check the usual rise in pressure from insufflations and when not functioning would permit it. Clotting was prevented by heparin.

Of 11 animals that gave bigeminal arhythmias from insufflations after connecting with the equalizer, six may be reported as yielding an earlier onset of the arhythmia when the rise in blood pressure was allowed, while in five the rise made no difference.

Comparisons of a large number of tracings in which the insufflations were preceded by a rise in blood pressure obtained mechanically with their corresponding control insufflation graphs, showed the mechanical rise to

be favorable for an earlier arhythmia in half of the comparisons and of no aid in the other half. Frequently these favorable records for an earlier onset of the arhythmia also revealed a longer duration of the arhythmia.

Elimination of the depressor nerves. In 7 acute experiments, 3 animals gave an earlier arhythmia, 4 showed no change, while 5 yielded a longer interval of arhythmia and 2 no change. In 4 animals in which insufflations were recorded both before and several days after sectioning the depressors, 2 animals gave an earlier and longer interval of arhythmia and 2 no change. Two animals in which all insufflation graphs were taken several days after cutting the depressors disclosed an earlier appearance and longer duration of the arhythmia when compared to a group of 20 controls in which the depressors were intact. Ordinarily sectioning the depressors caused hypertension and a faster pulse.

Elimination of the carotid sinuses. The right carotid sinus (carotid connected to the manometer) and bifurcations of the right external carotid were extirpated. The left sinus and bifurcations of the left external carotid were denervated by removal of the adjacent connective tissue and swabbing with alcohol, being careful not to injure any of the neighboring nerves. This procedure did not cause an earlier onset of the arhythmia from insufflations in 6 acute experiments, but did result in a longer interval of arhythmia in 4 animals. In one animal the duration of the arhythmia was the same and in another it was shorter. Tachycardia occurred from insufflations in one animal in which the carotid sinuses had been denervated for 2 days. Before stimulation this animal exhibited a marked hypertension and isolated ectopic beats.

Elimination of the depressor nerves and carotid sinuses. All experiments were acute. Since the rise in blood pressure which immediately followed this procedure was frequently sufficient to cause a bigeminal pulse for a minute or longer no insufflations were given during the following 15 minutes. A table of records from 6 animals shows the above procedure to be favorable for an earlier onset of the arhythmia in half of the animals while it produced no change in the other half. Five of the six animals, however, yielded a decidedly longer interval of arhythmia or several short intervals.

It occurred to the writer that the protective power of the depressors and carotid sinuses in holding back the arhythmia from hypertension might be further enhanced by taking blood pressure from the femoral artery instead of the carotid, thereby leaving both carotid sinuses to function to their maximum capacity. In a small series, a bigeminal pulse followed the first insufflation of benzol in half of the animals, which is well within the ratio reported for the first paper of this series where the tracings were recorded from the carotid.

Faradization of the depressor nerve before and during insufflation. In this experiment the left depressor was stimulated centrally with a strong

current (second coil at 5 to 4 cm.). Insufflations were started as soon as a drop in blood pressure was observed and both stimulations were continued for approximately the same time. A control insufflation record was taken before and after each test. Four animals may be reported as having one or more records in which the arhythmia from insufflations was completely blocked by depressor stimulation. One of these animals also responded by a delayed arhythmia. Four animals, counting the last mentioned, disclosed a pronounced delay (10 to 16 seconds); four revealed slight delays (3 to 6 seconds) and three little or no delay (0 to 2 seconds). Two of the last mentioned had high blood pressures (130 and 135 mm.) and a fast pulse.

Several insufflation graphs in which depressor stimulation prevented a bigeminal arhythmia revealed that the total rise in blood pressure was as great and sometimes greater than occurred in the control graphs in which insufflation produced arhythmias.

Faradization of the depressor nerve during benzol insufflation in double vagotomized rabbits. In 9 rabbits in which the left depressor was stimulated centrally after vagotomy 5 showed no delay, while 4 revealed some delay as shown by an increase of the latent period from 5 to 10, 10 to 17, 8 to 17 and 3 to 7 seconds. In 5 animals in which the effect of stimulating the depressor was tested before and after vagotomy, one can be reported as having about the same delay of the arhythmia after vagotomy as before, one revealed considerable delay before vagotomy and slight delay afterward, while three showed some delay before and no delay afterward. In these experiments the delay from depressor stimulation seemed to be in direct relationship to the time required for blood pressure to reach a certain necessary level to produce the arhythmia. The pulse excursions exhibited a slight or no perceptible slowing and strengthening of the excursions from depressor stimulation, but did show a moderate slowing and strengthening from the insufflations. There was seldom a lesser drop in blood pressure from depressor stimulation after vagotomy than before, which is in harmony with Porter and Bayer's results.

Faradization of the depressor nerve centrally during insufflations when blood pressure was maintained at a constant level. It was necessary in this experiment for the equalizer to take care of a drop in pressure as well as a rise. This was secured by having a limited flow of saline entering and leaving the outflow bottle, regulated constantly by an assistant to about half the capacity of the outlet. To determine whether the depressor and insufflation stimulations were working properly occasional tests were made with the equalizer closed. Control insufflations were recorded before and after each depressor test. Strong faradic currents (second coil 5 to 3 cm.) were used and clotting was prevented by heparin.

Three animals may be reported as giving records in which the onset of

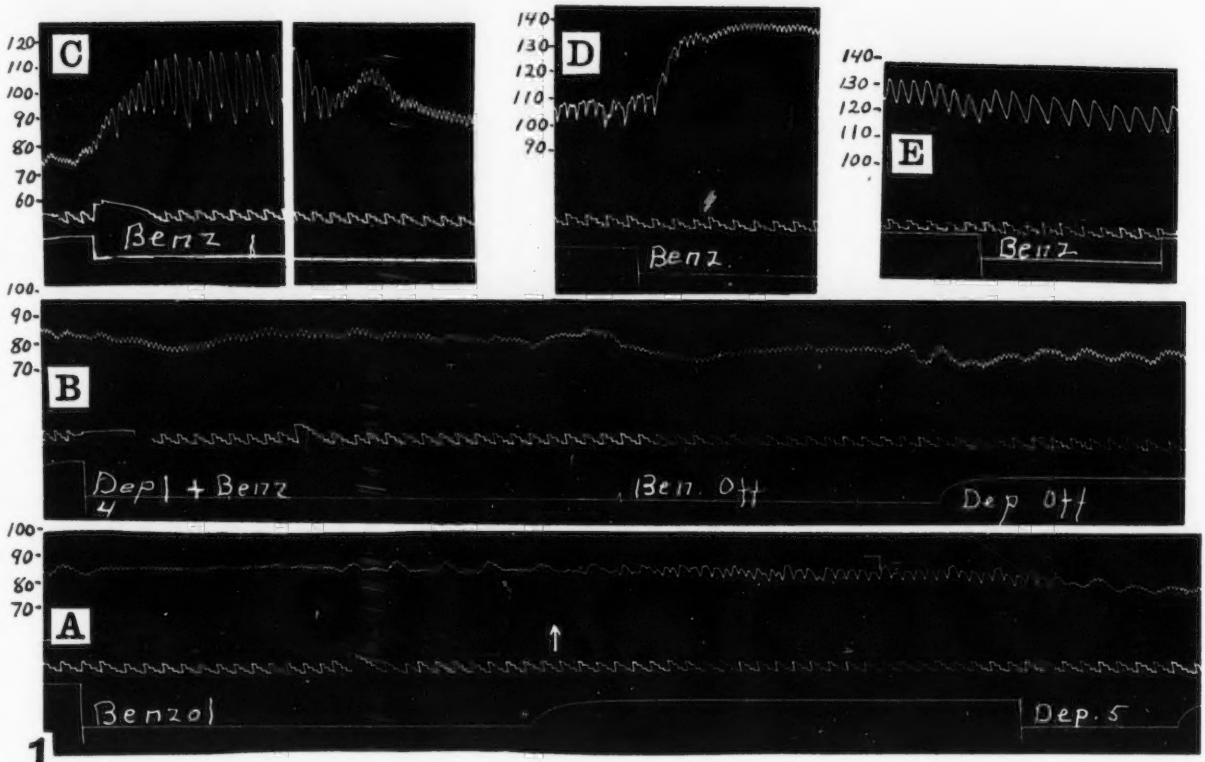


Fig. 1A. Carotid tracing of a bigeminal arhythmia obtained from benzol insufflation when an equalizer was attached to abdominal aorta; observe faradization of the depressor terminates arhythmia without drop in pressure, time in seconds.

B. Similar to A and next in sequence; note that faradization of the depressor nerve prevents a bigeminal pulse from benzol insufflation with little or no vascular changes except bradycardia, time in seconds.

C. Carotid tracing of a bigeminal arhythmia obtained from insufflation of benzol after adrenalectomy. Omitted portion represents a 38 second interval of arhythmia, time in seconds.

D. Carotid tracing showing an arrest of a bigeminal arhythmia from insufflation of benzol for 15 seconds (return to normal not shown) by a similar insufflation that caused a rise in blood pressure, time in seconds.

the arhythmia from insufflations was arrested for a time by central stimulation of the depressor nerve while blood pressure was maintained at approximately 85 mm. throughout. Tracing B (fig. 1) is illustrative of 2 animals in which the preceding and accompanying stimulations of the depressor nerve prevented a bigeminal arhythmia from insufflations with little or no alteration of blood pressure, when the preceding insufflation (fig. 1, A) and the following insufflation (not figured) produced arhythmias in the absence of depressor stimulation.

The first stimulation of the depressor nerve, with the equalizer functioning, slowed up the pulse sufficiently for benzol to give the arhythmia in one animal with a high blood pressure and fast pulse in which previous insufflations had produced only a tachycardia.

Double vagotomy. In 9 rabbits in which the last of several arhythmia records from insufflations taken before vagotomy was compared with a similar record after vagotomy, 5 disclosed no apparent change in the onset of the arhythmia, 3 revealed an earlier appearance of the arhythmia and in one it was delayed. Three of this series disclosed a longer interval of arhythmia after vagotomy, 2 a shorter interval and 4 no change. It is of interest to note that 3 animals of this series acquired one or more intervals of bigeminal pulse from the rise in blood pressure that followed vagotomy.

Numerous insufflation records taken directly after vagotomy, when compared to similar tracings from 20 animals having their vagi intact, indicate a tendency for an earlier onset and longer duration of the arhythmia following insufflations, but the difference is not marked in many tracings. The immediate effect of vagotomy is usually hypertension and tachycardia and for a time conditions are very favorable for an arhythmia from a rise in blood pressure, in fact the increased hypertension from vagotomy may result in one or more intervals of bigeminal pulse.

Koblanck and Roeder have reported that vagotomy prevented the arhythmia resulting from stimulation of the nasal septum for a time, but later it could be induced somewhat reduced. Levy found that vagotomy rarely favored a predisposition to arhythmias from compression of the aorta. It is of interest to note that Danielopolo, Marcou and Proca have recently claimed that the delayed extrasystoles obtained from stimulation of the carotid sinus after cutting the vagi or cardiac sympathetics were independent of the depressor vascular reflex.

Peripheral faradization of the vagus. Stimulating the left vagus with a weak current (second coil at 12 cm. and tilted) before and simultaneously with benzol insufflation resulted in the usual drop in blood pressure, bradycardia, little or no rise in pressure from the insufflations and no sign of premature systoles during the interval of stimulation.

In addition the effect of previous stimulations of the vagus with much stronger currents was studied. It resulted in delaying the arhythmia

D. Carotid tracing showing an arrest of a bigeminal arhythmia from insufflation of benzol for 15 seconds (return to normal not

from insufflations in half of the animals and in the other half it either made no difference or brought about an earlier appearance of the arhythmia. When favorable the vagus assistance was apparently due either to a necessity for a slowing of the pulse or else to a higher compensatory rise in blood pressure which when added to the rise from insufflations was an aid to the trigeminal impulses in producing the premature systoles.

Extirpation of the adrenals. The purpose of this experiment is to determine if the absence of simultaneous functioning of the adrenals caused any delay or shortening of the interval of bigeminal pulse from insufflation. Two procedures were utilized. One consisted of removing the adrenals separately on alternate days with one or two days intervening between the last excision and the tests. The other, which was more satisfactory, consisted of removing the right adrenal a day before the tests and the left one shortly before the tests. One or more insufflation tests were sometimes recorded before the last adrenal was removed. All animals were autopsied.

Three out of four animals in which the last adrenal was removed shortly before the tests responded with premature systolic arhythmias as quickly and as long after the last adrenal was removed as before. The first insufflation record from one of these adrenalectomized animals is shown in figure 1 C. The arhythmia appears within 3 seconds and continues for 50 seconds. (The space represents a 38 second interval of arhythmia purposely omitted.) One of the four animals suffered considerable shock from removal of the first adrenal and failed to respond with an arhythmia from insufflation with one adrenal intact.

Two animals in which the last adrenal was removed a day and two days respectively before the tests, failed to give a bigeminal pulse following insufflations. Blood pressure was much lower in these animals than in the previous set. Nevertheless insufflations caused considerable hypertension and bradycardia. In both animals intravenous injection of 0.25 cc. of (1:1000) epinephrin resulted in a short bigeminal arhythmia at the time of the slowing of the pulse, probably from the rise in pressure.

Slow pulse. The writer has a large number of records which indicate that the slowing of the pulse from insufflations is an aid to the trigeminosympathetic impulses in producing a bigeminal ventricular arhythmia. If it does not take place, the result is often a tachycardia. In some instances the desired slowing has been accomplished from peripheral stimulation of the vagus or central stimulation of the depressor.

EFFECT OF VARIOUS AGENCIES ON THE ARHYTHMIA CAUSED FROM INSUFFLATIONS. It is essential in this phase of the problem to use animals that yield reasonably long intervals of bigeminal pulse and to take a number of tests from each animal.

Agencies which cause a drop in arterial pressure. Under this head we

include peripheral stimulation of the vagus nerve, central stimulation of the depressor nerve (with and without an equalizer and vagotomized) and inhalation of amyl nitrate.

Stimulation of the left vagus peripherally with a weak faradic current for 2 to 5 seconds at the time of a bigeminal pulse elicited from insufflation caused a permanent cessation of the arhythmia in 2 animals and a temporary cessation in 7 animals. In the latter the arhythmia returned shortly after the compensatory rise in blood pressure had reached its maximum height. Mechanical stimulation of the peripheral end of the left vagus arrested the bigeminal arhythmia from insufflations for a short interval in 2 animals while it produced no effect in 3 animals.

Central stimulation (second coil 5 to 3 cm.) of the left depressor nerve permanently stopped the arhythmia from insufflations in 6 rabbits and arrested the arhythmia for some time in 5 rabbits. The early arhythmias of 5 rabbits were interrupted by depressor stimulation and the later ones were permanently stopped. Depressor stimulation stopped the arhythmia in one vagotomized rabbit, interrupted it in two and in two the early arhythmias were interrupted and the later ones were stopped. Depressor stimulation also interrupted the arhythmia in one animal in which the carotid sinus and depressor reflexes were eliminated. It is of especial interest that arhythmias from insufflations were permanently stopped by depressor stimulation when blood pressure was maintained at approximately 85 mm. by an equalizer. Figure 1 A serves as an example.

Numerous bigeminal arhythmias obtained from artificial rises in blood pressure have been permanently or temporarily arrested by stimulating the depressors or vagi.

Pressure on the abdominal wall, which of course may involve other factors than the ensuing drop in blood pressure, is a very efficient method of stopping or interrupting a bigeminal pulse from insufflations or from a rise in blood pressure. Bleeding will also accomplish the same result.

Arhythmias produced from insufflation and artificial rises in blood pressure were always stopped during the drop in blood pressure that followed inhalation of amyl nitrite.

The above effects of lowered blood pressure are in harmony with those Levy and Regniers obtained on premature systoles produced from compression of the aorta or carotid and drugs that caused vasoconstriction.

Agencies which cause a rise in arterial pressure. Under this heading will be included occlusion of the abdominal aorta, insufflation of benzol, and intravenous injection of epinephrin.

A rise in blood pressure from 125 mm. to 175 mm. from occlusion or pressure on the aorta arrested many arhythmias from insufflations for about 10 seconds in 4 rabbits. The same results were obtained in 3 animals in which the depressor nerves and carotid sinuses were eliminated

and in several vagotomized animals. A slight rise of a few millimeters was sufficient to arrest the arhythmia for 7 seconds in one animal. A considerable rise in blood pressure did not stop the bigeminal pulse in one animal, but changed the character of the excursions from "type I"[1] to "type II."

The effect or further insufflation of benzol on arhythmias obtained from benzol insufflation, mechanical stimulation of the nasal epithelium or mechanical rises in blood pressure was either an arrest of the arhythmia for a few seconds or an abrupt alteration of the type of the excursions. The latter may be a change from type I to type II or the reverse or it may consist of a lengthening or a widening of the excursions. Ordinarily the arhythmia was interrupted (fig. 1 D) in those records where it appeared late and blood pressure was about normal so that considerable rise took place. In other records in which the arhythmia came on earlier at the peak of the rise and insufflations produced little or no additional rise, the character of the excursions was altered (fig. 1 E). Here the excursions are greatly widened and may represent trigeminal beats. It should be noted that the above mentioned alterations of the excursions and interruptions of the arhythmia have been produced from insufflation of benzol after elimination of the depressors and carotid sinuses and after double vagotomy.

The effect of epinephrin on the arhythmia from insufflations was essentially the same for 8 rabbits when a dosage of 0.25 cc. of (1:1000) was used. In each instance the normal rhythm was regained with the rise in blood pressure and when the maximum rise was reached a few ectopic beats appeared at the point where they first appear in a normal adrenalin record. After eliminating the carotid sinuses and the depressors or the vagus nerves the injection of epinephrin was fully as effective and sometimes more effective for a longer arrest of the premature systoles.

Discussion. A comparison of the bigeminal arhythmia obtained from insufflations (trigemino-sympathetic impulses) with a similar arhythmia obtained from a pronounced rise in blood pressure discloses some differences. In an earlier paper the first mentioned arhythmia has been shown to depend entirely on the integrity of the cervical spinal cord and the cardiac sympathetics while the latter arhythmia does not depend on these structures. Some unreported experiments have demonstrated that the arhythmia from occlusion of the aorta or intravenous injection of adrenalin (arhythmia does not appear if hypertension is prevented) appeared as early and generally lasted longer in pithed and vagotomized animals. Whether these two bigeminal arhythmias are produced by the same peripheral myo-neural mechanism or whether one is of neurogenic origin and the other

[1] For example of type I bigeminal pulse excursions see (fig. 1 C) and for type II (fig. 1 D).

myogenic is not known. In 1900 Hering attributed the bigeminal pulse from a rise in blood pressure to myogenic changes produced through abnormal resistance to emptying of the ventricles.

After eliminating the vagus nerves or the depressors and carotid sinuses hypertension appears to be a considerable factor in the production of the arhythmia that follows insufflations, and in vagotomized animals the inhibitive action from stimulation of the depressors seems to be chiefly due to the drop in blood pressure and the delayed rise, though there may be some direct inhibitive action of the depressors on the cardiac sympathetics as has been pointed out by Brucke.

If Wollard's anatomical studies, which suggest that the auricles and auriculo-ventricular bundle are supplied by both sympathetics and parasympathetics and the ventricles are supplied only or chiefly by sympathetics, are confirmed, our interpretation of the neuro-physiology of the heart may have to be altered considerably. On this basis it might be possible to explain the origin of the ventricular premature systoles from a bombardment of the ventricles by a series of trigemino-sympathetic impulses. Central vagus, carotid sinus and other sensory stimulations should produce a like effect on the ventricles. Greene and Maneval's recent work on the action of certain myoneural drugs suggests that the sympathetics of the ventricle have a higher threshold than the sinus or the auricle. Nevertheless if the ventricles are only or chiefly innervated by sympathetics, a series of sympathetic impulses should initiate premature ventricular beats.

If the sympathetics to the heart are easily fatigued as was pointed out by Hunt many years ago and indicated by many of the writer's experiments, this fatigue may be an important agency in protecting the heart from premature systoles and tachycardia.

It is of interest to note that Bourne found alkalosis, intravenous injection of adrenalin, vagus stimulation were favorable factors for an increase of the ventricular ectopic beats caused from electrical stimulation; while anoxemia, compression of the aorta and stimulation of the accelerator nerves caused no effect. In his autogenic tests, raised blood pressure, anoxemia, adrenalin and acceleration were favorable for an increase in the number of ventricular ectopic beats, while vagus stimulation produced both an increase and a decrease, the general tendency being an increase.

It was rather striking that bigeminal arhythmias from insufflations or artificial rises in blood pressure can be stopped or altered by agencies that have a diametrically different mode of action on the vascular system, and even by the same agency that caused the original arhythmia. In many ways these results are in harmony with Foulger and Jackson's work on the premature systoles obtained from intravenous injection of barium chloride. The recent observation of Kouwenhoven, Hooker and Langworthy that a heart thrown into fibrillation by electrical stimulation can be restored to

normal rhythm by the same means seems to fall in the same general category.

There appears to be little danger of over-estimating the importance of a correct vago-sympathetic balance for the preservation of a uniform cardiac rhythm.

SUMMARY

Double vagotomy, elimination of the depressor nerves either separately or combined with elimination of the carotid sinuses, and the hypertension from insufflations produced an earlier onset and longer duration of the arhythmia resulting from insufflations of benzol (trigemino-sympathetic impulses) in approximately half or more of the animals.

Central faradization of the left depressor generally delayed or prevented the arhythmia from insufflations, but hastened it in a few animals in which the pulse was fast. Depressor stimulation also produced the same effect when blood pressure was maintained at approximately the same level by an equalizer. It likewise delayed the onset of the arhythmia in half of the vagotomized animals.

Peripheral faradization of the left vagus at the time of insufflation prevented the arhythmia during the interval of faradization, while previous faradization often delayed the arhythmia but frequently hastened it in animals having a fast pulse and a high compensatory rise in blood pressure.

The absence of simultaneous functioning of the adrenals caused no delay or shortening of the period of the arhythmia from insufflation.

Regniers' claim that the carotid sinus and depressor nerve endings afford protection against premature systoles from hypertension is confirmed for a small or moderate rise.

Arhythmias from insufflations appeared as early and lasted as long when both carotic sinuses were intact as when only one was functioning.

Attention was called to Wollard's observation that the ventricles seem to be innervated chiefly by sympathetics and a suggestion was made as to the possibilities of such a mechanism for instigating ventricular premature beats.

Premature systolic arhythmias produced from insufflations or a rise in blood pressure were terminated or interrupted by agencies which cause a drop in blood pressure, while they were generally only interrupted or altered by agencies which cause a rise.

Assistance for these experiments was financed by a gift to the school by the General Education Board.

BIBLIOGRAPHY

Bourne, G. 1927. Amer. Heart Journ., iii, 51.
Brücke, E. T. 1917. Zeitschr. Biol., lxvii, 507.
Danielopolu, Marcou et Proca. 1931. C. R. Soc. Biol., cvi, 962.

FOULGER AND JACKSON. 1929. Abst. 13th Internat. Physiol. Cong., 84.
GREENE AND MANEVAL. 1932. This Journal, ci, Proc., 43.
HEIDENHEIN, R. 1872. Pflüger's Arch., v, 143.
HERING, H. E. 1900. Pflüger's Arch. lxxxii, 1.
1927. Die Karotissinusreflexe.
HEYMANS, C. 1929. Le sinus carotidien.
HUNT, R. 1899. This Journal, ii, 395.
KISCH, B. 1921. Münch. med. Wochenschr., lxviii, 395.
KOBLANCK UND ROEDER. 1908. Pflüger's Arch., cxxv, 377.
KOCH. 1931. Die Reflektorische Selbsteuerung des Kreislaufes.
KOUWENHOVEN, HOOKER AND LANGWORTHY. 1932. This Journal, ci, Proc., 65.
LEVY, A. G. 1913. Heart, iv, 319.
1914. Heart, v, 299.
PORTER AND BEYER. 1901. This Journal, iv, 283.
REGNIERS, P. 1929. C. R. Soc. Biol., ci, 159.
1930. Arch. Intern. Pharm. Thérap., xxxix, 371.
WOLLARD, W. H. 1926. Journ. Anat., lx, 345.

STUDIES ON THYROGLOBULIN

II. Absorption of Thyroglobulin and Related Substances from the Alimentary Canal

B. O. BARNES[1] AND J. G. BUENO

From the Physiological Laboratory of the University of Chicago

Received for publication November 25, 1932

Hektoen, Kanai and Dragstedt (1), using the precipitin method, reported that thyroglobulin could be detected in the blood after ingestion of fresh thyroids. Since this would require some thyroglobulin being absorbed undigested and since Barnes, Carlson and Riskin (2) observed that the digestive enzymes rapidly break down thyroglobulin, it seemed advisable to measure quantitatively the rate of absorption in the absence of digestion.

Methods. Soluble thyroglobulin has been employed, prepared by the method of Barnes (3). The solution whose iodine content was known was introduced into closed loops of intestine in dogs under light barbital anesthesia, and into Thiry-Vella fistulas in normal dogs. Two closed loops were used in the anesthetized dogs, one starting about a foot below the pancreas and extending downward for about 60 cm., while the other began about a foot above the appendix and extended upward for 50 to 60 cm. The loops were washed with warm saline prior to the introduction of the material and again two or three times at the end of the experiment. The Thiry-Vella fistulas were prepared about one foot above the appendix and were about 10 inches long. The two ends were brought out on either side of the dog as high up on the abdominal wall as possible, such that when the dog was standing normally, none of the fluid in the lumen would run out. Fluid could be introduced or withdrawn by inserting into either end a rubber tube attached to a syringe. Control experiments showed that if the fluid was withdrawn at once, about 98 per cent of the iodine could be recovered. Iodine estimations were carried out by Kendall's method (4). Other compounds including potassium iodide, thyroxine, and split products of thyroglobulin obtained by treating the protein with pepsin were employed. Pepsin was allowed to act for 12 hours and upon bringing the pH to 5.0 about half of the iodine in thyroglobulin was precipitated. Both the filtrate and the dissolved precipitate were tested for absorbability.

Results. In the acute experiments 13 dogs have been used, thyro-

[1] National Research Fellow in Physiology.

TABLE 1

Showing the absorption of thyroglobulin, thyroxine, potassium iodide, and the two fractions of iodine obtained by bringing digested thyroglobulin to pH 5.0

(Length of experiments, 3 hours)

DOG	SEGMENT	I_2 INTRODUCED	I_2 ABSORBED	PREPARATION
		mgm.	*mgm.*	
1	Duodenum	3.1	2.0	Thyroglobulin
	Ileum	4.7	1.0	Thyroglobulin
2	Duodenum	2.6	2.0	Thyroglobulin
	Ileum	2.6	2.0	Thyroglobulin
3	Duodenum	14.9	6.7	Thyroglobulin
	Ileum	19.9	7.8	Thyroglobulin
4	Duodenum	14.9	5.7	Thyroglobulin
	Ileum	19.9	4.3	Thyroglobulin
5	Duodenum	24.9	15.6	Thyroglobulin
	Ileum	24.9	10.6	Thyroglobulin
6	Duodenum	14.9	6.7	Thyroglobulin
	Ileum	17.4	3.7	Thyroglobulin
7	Duodenum	8.0	1.4	Pepsin filtrate
	Ileum	8.0	0.6	Pepsin filtrate
8	Duodenum	5.2	2.0	Pepsin filtrate
	Ileum	5.2	1.7	Pepsin filtrate
9	Duodenum	9.0	1.7	Pepsin precipitate
	Ileum	18.0	7.1	Pepsin precipitate
10	Duodenum	8.4	3.0	Pepsin precipitate
	Ileum	12.2	3.3	Pepsin precipitate
11	Duodenum	6.3	1.5	Pepsin precipitate
	Ileum	5.5	3.6	Pepsin precipitate
12	Duodenum	13.0	11.6	Thyroxine
	Ileum	41.0	36.0	Potassium iodide
13	Duodenum	13.0	8.8	Thyroxine
	Ileum	26.0	12.0	Thyroxine

globulin being introduced into 6, split products of thyroglobulin into 5, and KI and thyroxine into the other 2. Table 1 summarizes the results.

It is apparent in the first six animals that the iodine in thyroglobulin disappears rapidly from the gut. Considerable variation is found which

is related in part to the length of the segment, the amount introduced, the depth of the anesthesia, etc. The thyroglobulin used contained about 0.7 per cent iodine, therefore in some cases between 1 and 2 grams of thyroglobulin were absorbed in the 3-hour interval. No attempt was made to compare the rate of absorption in the two segments, their lengths and the quantity of iodine introduced were frequently different. It was necessary to determine the state of the iodine remaining in the intestine to be sure that surface enzymes on the wall of the gut had not digested the thyroglobulin. This was done by mixing the recovered solution with an equal volume of saturated ammonium sulfate. Practically all of the iodine was precipitated indicating that digestion had not occurred.

Dogs 7 and 8 show the results obtained when the acid soluble iodine from peptic digestion was introduced while dogs 9, 10 and 11 were used for the acid insoluble fraction. These five dogs show a decreased rate of absorption from those in which the whole thyroglobulin was tested. Since the digested fractions contain much smaller molecules the experiments indicate that the intestinal epithelium is more permeable to thyroglobulin. Dogs 12 and 13 were added to the list because of the reports that thyroxine is much less effective by mouth. Prior to the recent work of Schittenheim and Eisler (5) it was not known whether some thyroxine was not absorbed or whether it was partly destroyed in the alimentary canal. Their experiments indicated that it was absorbed from a closed loop and our work confirms their observations. They used only 10 mgm. doses while one dose of 40 mgm. and two of 20 mgm. have been employed here. In dog 12, potassium iodide was used as a basis of comparison and it can be seen that the inorganic iodine is much more rapidly absorbed. In order to make sure that the thyroxine was not adherent to the mucosa, the segments were washed with dilute alkali, the mucosa stripped off and extracted. The amount of iodine found was included in that recovered from the lumen. It seems clear that thyroxine is absorbed from closed loops of intestine.

The experiments on unanesthetized dogs using the Thiry-Vella fistulas showed a more rapid rate of absorption of thyroglobulin than the acute experiments. Frequently the loops would contract and spill some of the fluid, but 7 successful experiments on one dog and 3 on another showed a rate of absorption of the whole protein two or three times faster than the anesthetized dogs.

Since the above experiments indicated that thyroglobulin was absorbed rather rapidly, a final test was made using anaphylaxis as an indicator. Six guinea pigs were sensitized to thyroglobulin by a single subcutaneous injection. Two weeks later under light ether anesthesia, the intestines were exposed and a solution of thyroglobulin injected into the lumen through a hypodermic needle. A ligature was tied around the gut behind the injection to prevent the escape of fluid into the abdomen. The gut was re-

placed, the wound quickly closed and the animal was soon out of the anesthesia. A control was subjected to the same operative procedure and saline was injected. Two pigs showed severe shock about thirty minutes after the injection, the symptoms including urination, defecation, sneezing, coughing, difficult respiration, and loss of equilibrium. One animal died in convulsions $1\frac{3}{4}$ hours after the injection and the other died some hours later. Two others injected with thyroglobulin showed less severe symptoms of anaphylaxis while another and the control showed no effects. These experiments may be taken to indicate that thyroglobulin was absorbed undigested since in a previous experiment it was found that thyroglobulin digested with pepsin would not cause anaphylaxis after subcutaneous injection.

Very little work has been done with soluble thyroglobulin and very few of its properties are known. These experiments might suggest that the whole protein is capable of penetrating cell membranes but this possibility will have to be investigated further.

SUMMARY

It has been found that thyroglobulin is absorbed from closed loops of intestines in anesthetized dogs and more rapidly from Thiry-Vella fistulas. Thyroxine is also absorbed but not as rapidly as KI. Anaphylactic symptoms were observed in guinea pigs sensitized against thyroglobulin when this protein was introduced into the gut. These experiments indicate that thyroglobulin can be absorbed undigested.

This problem was suggested by Dr. A. J. Carlson to whom we are greatly indebted.

BIBLIOGRAPHY

(1) Hektoen, Kanai and Dragstedt. Journ. Amer. Med. Assoc., 1925, lxxxiv, 114.
(2) Barnes, Carlson and Riskin. This Journal, 1931, xcviii, 86.
(3) Barnes. Proc. Soc. Exp. Biol. and Med., 1931–32, xxix, 680.
(4) Kendall. Journ. Biol. Chem., 1914, xix, 251.
(5) Schittenheim and Eisler. Deutsch. Ges. Exp. Med., 1932, lxxx, 568.

CHEMICAL OBSERVATIONS ON FLUIDS OF THE SEMINAL TRACT

I. Inorganic Phosphorus, Calcium, Non-Protein Nitrogen and Glucose Content of Semen and of Seminal Vesicle, Prostate and Spermatocele Fluids in Man

C. B. HUGGINS and ANNA A. JOHNSON

From the Department of Surgery of The University of Chicago

Received for publication November 21, 1932

Investigation of the seminal tract fluids has been greatly neglected by chemists, although this biological system is of interest because of its relationship to sperm cells and fertilization, and because it is profoundly influenced by endocrine products and is the seat of certain hypertrophies and hypoplasias whose exact pathogenesis is unknown. In this study, data have been obtained by the application of some of the present biochemical methods to human seminal tract fluids in order to establish normal results as a basis for further comparison: this has been necessary because of the well known species differences in semen. The seminal fluid has been integrated as far as possible: an interesting and unique distribution of some of the commoner ions appeared during the study, which throws light on the functions of the accessory glands.

Results of previous investigations. An exceedingly scant literature exists on quantitative chemical studies of seminal fluids. The analyses reported are frequently few in number, incomplete and difficult to interpret.

Semen. The earliest work on the chemistry of washed spermatozoa heads was done by Miescher (1) and Schmiedeburg (2). Additional workers in this field, which has been more extensively studied than any other part of the seminal tract, have been A. P. Matthews (3), Burian (4), Steudel (5) and Lynch (6). According to these workers, the defatted spermatozoa heads consist almost entirely of a combination of a basic protein (protamin) and certain inorganic bases with nucleic acid. Lynch (6) found that all of the phosphorus in spermatozoa was combined in nucleic acid; Fourcroy and Vauquelin (7) and Slowtzoff (8) found large quantities of phosphorus in ashed semen. The latter found an average of 0.9 per cent ash of which 15.08 to 22.4 per cent was CaO and 20.55 to 36.04 per cent was P_2O_5. The results of determinations of specific gravity, water content and ash reported by the latter group of workers and by Kölliker (9) and Lode (10) are widely divergent.

Prostatic-seminal vesical secretions. The most comprehensive chemical study of the fluids of the seminal tract apart from spermatozoa was reported by McCarthy, Stepita, Johnstone and Killian (11), who combined the fluid expressed from these structures in vivo, and found in general "an unusually high concentration of calcium, inorganic phosphorus, hydrolyzable phosphorus and amino nitrogen." The sugar content showed marked variation. Large variations between maximum and minimum determinations were reported by them and these may be explained, at least in part, below.

Seminal vesicle secretion. In a large percentage of adult human males the seminal vesicle contains spermatozoa (Brack, 12, Lit.). Histological evidence (Akutsu, 13, Kölliker, Brack) suggests a secretory function of the vesicular epithelium. Chemison is said to have reported (Brack) that the ash of the fluid contents is rich in phosphorus. The pH of the fluid in guinea pigs is recorded by Armitstead (14), using a colorimetric method, as 7.6. The fluid in guinea pigs contains large amounts of protein resembling fibrinogen (Landwehr, 15) which coagulates on the addition of blood plasma or the secretion of a specific coagulating gland in the sexual system (Walker, 16).

Prostate secretion. Difficulty is encountered in harmonizing the pH studies made by Muschat (17), Mettenleiter (18) and Sergijewsky and Bacromejew (19). Muschat reports a pH range of 7.0 to 7.4 (average 7.24) in normal adult humans. Karassik (20) reported that dog prostatic fluid did not reduce Fehling's or Benedict's solutions and that a diastase was present in both dog and human fluids. A pressor substance is present in extracts of the prostate (Biedl, 21; Collip, 22).

Spermatocele. (A chronic, sterile, non-inflammatory cyst of the epididymis whose milky white, odorless fluid contains spermatozoa.) The most complete studies of the fluid are by Ultzmann (23) and Hammarsten (24). Ultzmann found that the specific gravity varied from 1.002 to 1.009; water content 98.5 per cent; NaCl 0.71 to 0.86 per cent; albumin 0.2 to 0.5 per cent, traces of phosphorus and sulfur. Similar specific gravity figures have been reported by Hochenegg (25) and others. Vertun (26) found that the fluid contained 0.44 per cent protein and traces of NaCl and phosphorus but no spermine. Posner (27) similarly reports absence of spermine.

METHODS. *Biological.* Spermatocele fluid was collected under oil from the cysts by needle puncture with aseptic precautions. The other fluids studied were obtained after they had traversed the urethra: in each case the patient had urinated at least 30 minutes before the collection of fluid and the urethra was dried by gentle massage in the perineum. Contamination with urethral secretions and retained urine is a source of error in estimations on fluids obtained in this way, but gross contamination must certainly be minimal, since irrigation of the urethra with Ringer's solution showed that only traces of dissolved substances were added. Semen was obtained by ejaculation into clean glassware and usually 3 to 6 cc. were obtained. Prostatic

fluid (0.3–1.5 cc.) and seminal vesicle fluid (0.8–1.3 cc.) were secured by digital massage of these structures per rectum. The viscid yellowish seminal vesicle fluid could be obtained in approximately 5 per cent of men, who were usually young men with distended vesicles. All fluids were obtained from men known from previous examination to be free from disease of the genito urinary-tract. Microscopic examination was carried out on the fluids in all cases and those fluids which contained blood or pus were discarded. Microscopic examination was also used to detect seminal vesicle fluid, in examination of prostatic secretion. Care was taken in obtaining seminal vesicle fluid that the prostate gland was not emptied.

Chemical. Precautions were taken to reduce the loss of sugar due to glycolysis, and enzymatic action on the organic phosphates by the following procedures. Samples for sugar determinations were collected directly into calibrated tubes containing sulfuric acid. The amount was noted and the required amounts of sodium tungstate and additional sulfuric acid were immediately added. Samples for phosphate determinations were similarly collected into tubes containing trichloracetic acid. When pH determinations were to be made on semen, paraffin oil was added to the fluid immediately after ejaculation. Calcium was estimated by the Clarke-Collip (28) method or the incineration procedure of Fiske (29); acid soluble and total phosphorus by the method of Fiske and Subbarow (30); reducing substances by the method of Folin (31); glucose by the yeast fermentation method of Hiller, Linder and Van Slyke (32); chlorides by the Whitehorn method (33); NPN by the Koch-McMeekin procedure (34); CO_2 constant by the method of Van Slyke and Neill; pH by the method of Hastings and Sendroy (36).

In the inorganic phosphate determinations in semen, prostatic and seminal vesicle fluids, an interfering turbidity appeared on adding the molybdic acid reagent to the trichloracetic acid filtrate, which was removed by ultrafiltration of this filtrate through a collodion membrane under a negative pressure of 150 mm. Hg. This turbidity did not occur in spermatocele fluid.

RESULTS AND DISCUSSION. *Spermatocele.* The results of the analyses of spermatocele fluid are given in table 1. The striking points are the total absence of acid soluble phosphorus and the negligible amount of sugar present. To account for this, the fluid accompanying the spermatozoa from the testicle into the spermatocele cysts either contains no phosphate, or it is removed by absorption or organic synthesis. Analysis of the fluid for its total phosphorus content revealed the presence of only 1.0 to 1.8 mgm. per 100 cc., which eliminates the last possibility from serious consideration. This finding harmonizes well with the observation of Lynch (6) that washed spermatozoa heads contain no inorganic phosphorus. A comparable situation has been encountered by Phemister, Hastings and Day (37) who found that the fluid present in the gall bladder whose duct is occluded contains very little phosphate. That absorption from spermatocele cysts can take place has been demonstrated in the case of dyes by Huggins and Entz (38). They found that after the injection of phenolsulfonephthalein and sodium indigotate into these cysts, these dyes appeared in the urine. However, in the absence of information regarding the phosphate content of the fluid coming directly from the duct system of the testis, no definite statement can be made regarding the reason for its absence in spermatocele fluid.

The calcium concentrations varied from 7.9 to 11.0 mgm. per 100 cc. of fluid. In view of the low protein content of the fluids (all except one contained less than 1.0 per cent) these calcium concentrations are exceptionally high for biological fluids. For example, if one deducts the calcium

TABLE 1

Spermatocele fluid

SUBJECT	pH	NON-PROTEIN N	PROTEIN	CO_2	Cl	GLUCOSE	Ca	INORGANIC P	SPECIFIC GRAVITY
		mgm./100 cc.	*gm./100 cc.*	*mM./L*	*mM./L*	*mgm./100 cc.*	*mgm./100 cc.*		
1	7.7	26.1	0.19	2.6	137.7		8.8	0.0	1.003
2	7.0	25.0	0.16	2.5			9.1	0.0	
3		25.5	0.20				8.8	0.0	
4		27.2	0.75				11.0	0.0	
5	7.2	21.4	0.40	6.7			10.0	0.0	
6	7.06	24.6	0.41	13.9			9.2	0.0	
7	6.91	22.2	2.17	3.9		Trace	10.06	0.0	1.002
8	6.91	28.8	0.13	8.4	139.8	Trace	9.0	0.0	1.002
9					130.7		9.4	0.0	
10					149.7		10.0	0.0	
11		30.0			145.3	0.0	9.6	0.0	
12					141.2		8.5	0.0	
13					125.2		8.1	0.0	
14							7.9	0.0	

TABLE 2

Semen

SUBJECT	pH	NON-PROTEIN N	CO_2 CONTENT	Cl	REDUCING SUBSTANCES	GLUCOSE	Ca	INORGANIC P
		mgm./100 cc.	*mM./L*	*mM./L*	*mgm./100 cc.*	*mgm./100 cc.*	*mgm./100 cc.*	*mgm./100 cc.*
1	7.23	55.0			325.2			
2	7.35	64.0		46.6	560.0		21.7	53.33
3	7.21				432.0	369	32.8	100.0
4	7.41				400.0	350	22.7	92.4
5	7.25		14.5		392.1	297		80.0
6	7.12				364	332	11.87	70.4
7	7.3		11.5		312.0	221.6	20.6	55.0
8		80.0	17.7	39.0	406.0		20.0	72.72
9	7.45			42.2	380.0	203		
10	7.05						16.0	66.8

bound to the proteins of the serum which are normally about 7 grams per 100 cc., the residual calcium is approximately 6 mgm. per 100 cc. This is much lower than the concentrations found in these fluids. The fact that the calcium does not precipitate is probably due to the fact that in most instances the pH and CO_2 content were low.

It is also of interest that the chlorides are relatively high for a biological fluid. The bicarbonate is low, however, and preliminary freezing point determinations indicate that the fluids are isosmotic with other body fluids.

Semen. The analytical results on semen are given in table 2. When compared with spermatocele fluid, the semen has a high phosphate content, a high glucose content and a lower chloride content. Freezing point determinations on normal semen (−0.56°, −0.58°, −0.61°) showed no essential difference in their osmolar concentrations.

Bilateral ligation of the vas deferens, as anticipated, did not change materially the concentrations of these substances (table 3) or the amount of semen and is proof that the composition of the semen is largely determined by the accessory sex system below the epididymis.

TABLE 3

Influence of bilateral ligation of vas deferens on semen

SUBJECT	NON-PROTEIN N	REDUCING SUBSTANCES	GLUCOSE	Cl	Ca	INORGANIC P
	mgm./100 cc.	*mgm./100 cc.*	*mgm./100 cc.*	*mM./L*	*mgm./100 cc.*	*mgm./100 cc.*
1 Before vasotomy	80.0	337	292	45.6	22.0	80
1 After vasotomy	114.0	333		38.8	25.0	70
2 After vasotomy					21.6	91.4
3 After vasotomy	78.0	363	331	26.7		66.6
4 After vasotomy	90.0	470	407	18.5		80.0
5 After vasotomy						76.0

Prostatic fluid. The results of analyses of prostatic fluid are shown in table 4. This fluid differs from semen in at least two important respects. First, the phosphate concentration is approximately that of blood serum; secondly, reducing substances are present in exceedingly low concentration.

Seminal vesicle fluid. The results of analyses of seminal vesicle fluid are given in table 5. In these fluids, the phosphate, reducing substances, non-protein nitrogen and chlorides were approximately the same as in semen and in seminal fluid after vas ligation. This is strong evidence that the amount of these substances in semen is determined by the seminal vesicle epithelium in view of the fact that the prostate gland and seminal vesicle are the major parts of the accessory sex apparatus and the niveau of these substances in prostatic secretion is markedly different from that in the seminal vesicle.

It is evident from these studies that discharged spermatozoa not in the seminal vesicle are exposed to a sudden increase in the amount of acid soluble phosphorus and glucose in their environment at the moment of ejaculation.

TABLE 4
Prostate fluid

SUBJECT	NON-PROTEIN N	REDUCING SUBSTANCES	Cl	INORGANIC* P
	mgm./100 cc.	*mgm./100 cc.*	*mM./L*	*mgm./100 cc.*
1	47.0	20	67.7	5.0
2	57.0	30	38.8	2.99
3	44.4	18		3.1
4	60.0	trace	39.0	4.4
5	33.3	trace		2.5
6	22.0	trace	80.0	3.38
7	65.0	19	39.4	3.94
8	85.0	48	98.8	2.4
9	175.0			2.0
10		40		3.4
11		21		3.2
12		trace		5.5
13		trace		3.72
14				4.17
15				3.12
16				2.0

* Fluid for inorganic P determinations collected and analyzed usually 1 week before or after other determinations were made.

TABLE 5
Seminal vesicle fluid

SUBJECT	NON-PROTEIN N	REDUCING SUBSTANCES	Cl	INORGANIC* P
	mgm./100 cc.	*mgm./100 cc.*	*mM./L*	*mgm./100 cc.*
1	109.7	358.4		30.3
2	80.0	275.0	29.8	56.0
3	90.0	275.0	30.4	48.8
4	98.0	625.0	16.1	61.5
5	87.0	417.0	35.5	35.0
6				52.6
7				30.0

* Fluid for inorganic P determinations collected and analyzed usually 1 week before or after other determinations were made.

The great variations in the findings reported by McCarthy and co-workers (11) for "prostato-vesicular secretion" may be explained in part by varying amounts of the secretions of these glands obtained for their studies, since

there is a marked chemical difference in the prostatic and vesicular components, and it would be difficult to obtain consistent results by combining them.

CONCLUSIONS

Spermatocele fluid contains no acid soluble phosphorus or glucose.

Semen contains large amounts of glucose, calcium, acid-soluble phosphorus and relatively small amounts of chloride.

These findings in semen are not appreciably lowered after eliminating testicular and epididymal excretions by vasotomy.

The seminal vesicle contains approximately the same amounts of these substances as semen, and evidence is presented that this structure is the chief source of origin in the semen of glucose, and acid soluble phosphorus.

Prostatic fluid contains very small amounts of reducing substances and glucose. The acid soluble phosphorus level here is about the same as in the blood serum.

At ejaculation, the environment of discharged spermatozoa, not present in the seminal vesicle, is rapidly altered by an increase in glucose and acid soluble phosphorus.

BIBLIOGRAPHY

(1) Miescher, F. Ber. d. deutsch. chem. Gesell., 1874, vii, Part 1, 376.
(2) Schmiedeburg, O. Arch. f. exper. Path. u. Pharm., 1900, xliii, 57.
(3) Matthews, A. P. Zeitschr. f. Physiol. Chem., 1897, xxiii, 399.
(4) Burian, R. Ergebn. d. Physiol., 1904, iii, 48.
(5) Steudel, H. Zeitschr. f. Physiol. Chem., 1911, lxxii, 303; lxxiii, 471.
(6) Lynch, V. Journ. Biol. Chem., 1920, lxiv, 319.
(7) Fourcroy and Vauquelin. Quoted by R. Burian, loc. cit.
(8) Slowtzoff, B. Zeitschr. f. Physiol. Chem., 1902, xxxv, 358.
(9) Kölliker, A. Zeitschr. f. Wissen. Zool., 1855, vii, 201.
(10) Lode, A. Pflüger's Arch., 1891, l, 278.
(11) McCarthy, J. F., C. T. Stepita, M. B. Johnston and J. A. Killian. Journ. Urol., 1928, xix, 43.
(12) Brack, E. Zeitschr. f. Urol. Chir., 1923, xii, 403.
(13) Akutsu. Arch. f. path. Anat. u. Physiol., 1902, clxviii, 467.
(14) Armitstead, R. B. Journ. Exp. Zool., 1924, xli, 215.
(15) Landwehr, H. A. Pflüger's Arch., 1880, xxiii, 538.
(16) Walker, G. Bull. Johns Hopkins Hosp., 1910, xxi, 182, 185.
(17) Muschat, M. Journ. Urol., 1926, xv, 593.
(18) Mettenleiter, M. Arch. f. Gynak., 1925, cxxvi, 250.
(19) Sergijewsky, M. W. and J. R. Bachromejew. Zeitschr. f. d. gesammt. exper. Med., 1930, lxxi, 303.
(20) Karassik, W. M. Zeitschr. f. d. g. exper. Med., 1927, liii, 734.
(21) Biedl, A. Innere Sekretion, 1913, ii, 343. Urban & Schwartzenberg, Berlin.
(22) Collip, J. B. Trans. Roy. Soc. Canada, 1929, xxiii, part 2, 165.
(23) Ultzmann, R. Wien. med. Presse, 1871, xii, 217, 247.
(24) Hammarstein, O. Quoted by Hochenegg.
(25) Hochenegg, J. Med. Jahrb., (Wien), 1885, 182.

(26) VERTUN, M. Centralb. f. d. med. Wissenschr., 1899, xxxvii, 529.
(27) POSNER, C. Centralb. f. d. med. Wissenschr., 1892, xxx, 225.
(28) CLARKE, E. P. AND J. P. COLLIP. Journ. Biol. Chem., 1925, lxiii, 461.
(29) HAMILTON, B. Journ. Biol. Chem., 1925, lxv, 101.
(30) FISKE, C. H. AND Y. SUBBAROW. Journ. Biol. Chem., 1925, lxvi, 375.
(31) FOLIN, O. Journ. Biol. Chem., 1929, lxxxii, 83.
(32) HILLER, A., G. C. LINDER AND D. D. VAN SLYKE. Journ. Biol. Chem., 1925, lxiv, 625.
(33) WHITEHORN, J. C. Journ. Biol. Chem., 1921, xlv, 449.
(34) KOCH, F. C. AND T. L. MCMEEKIN. Journ. Amer. Chem. Soc., 1924, lvi, 2066.
(35) VAN SLYKE, D. D. AND J. M. NEILL. Journ. Biol. Chem., 1924, lxi, 523.
(36) HASTINGS, A. B. AND J. SENDROY. Journ. Biol. Chem., 1924, lxi, 695.
(37) PHEMISTER, D. B., A. B. HASTINGS AND L. F. DAY. Ann. Surg., Oct. 1932 (in press).
(38) HUGGINS, C. B. AND F. H. ENTZ. Journ. Urol., 1931, xxv, 447.

A NEUROMUSCULAR MECHANISM IN THE DUCTUS EPIDIDYMIDIS AND ITS IMPAIRMENT BY SYMPATHETIC DENERVATION

F. A. SIMEONE[1]

From the Laboratories of Physiology in the Harvard Medical School

Received for publication November 29, 1932

The action of a neuromuscular mechanism in the forward movement of spermatozoa in the epididymis was first suggested by Moore and Quick (1924) in their work on vasectomy. The same mechanism has been postulated (Young and Simeone, 1930) for vasectomized guinea pigs in which the epididymides had been separated from the testes by ligature at the head of the epididymis. More recently, Toothill and Young (1931) have suggested a peristalsis "especially in the more distal portion of the epididymis" as one of the factors responsible for the distal progress of spermatozoa through this organ. Oslund (1928), on the other hand, considers the outward movement of seminal fluid as due almost exclusively to the pressure produced by the testis as a result of its own activity.

The work cited above, with the exception of that of Oslund, indicates that a neuromuscular action is of considerable importance in the function of the epididymis. Most suggestive are the experiments of Toothill and Young (1931), who found a distal movement of the contents of the epididymis even after this organ, by a ligature at its head, had been separated from the testis and ductuli efferentes. Excluding the possible action of the epididymal secretion, which is more intense proximally than distally (Benoit, 1926), the movement observed could be attributed only to a peristaltic action on the part of the epididymis distal to the ligature.

Anatomical evidence is not inconsistent with the postulated muscular activity in the ductus epididymidis. Ample provision for such action is made by the presence of circular smooth muscle fibers throughout its length, starting as a single layer in the ductuli efferentes and gradually increasing in amount until at the ductus deferens they are reinforced by longitudinal fibers to form a powerful muscular coat.

In spite of the suggestions from the different sources cited above regarding the possibility of muscular action on the part of the epididymis, this factor in the distal progress of the epididymal contents has as yet received

[1] Holder of a Bullard Fellowship, Harvard Medical School.

no direct attention experimentally. The experiments reported in this paper are the result of an attempt to demonstrate the existence of a neuromuscular mechanism by modifying its action through the agency of sympathetic denervation. The guinea pig was chosen for this work because it is a convenient laboratory animal especially suitable for this type of experimentation and because it has been the experimental animal in most of the former work.

METHODS. Attention was directed first to an investigation of the normal rate at which the contents of the epididymis pass distally. For this purpose the heads of the epididymides in five normal adult males were injected with minute quantities of Higgins' water-soluble India ink diluted with twice its volume of mammalian Ringer solution (without glucose).

The technique used was essentially that described by Toothill and Young (1931). The operations were performed with aseptic precautions under ether anesthesia. The animals with adequate diet were kept isolated in cages and their weights were recorded daily. In most cases, the animals began to increase in weight on the second day following the operation after a negligible loss in weight on the first day.

Three of the guinea pigs were autopsied fourteen days subsequent to the injection. A fourth animal was autopsied on the sixteenth day; and the fifth, after an examination of the epididymides by means of celiotomy on the fourteenth day, was autopsied on the nineteenth day after the injection. The results obtained agreed essentially with those recently reported by Toothill and Young, and for this reason it was thought unnecessary to accumulate more data on this point.

The remainder of the experiments concerned the effect of sympathetic denervation on the distal movement of the contents of the epididymis. From the generally accepted views regarding the innervation of the internal genitalia (Gaskell, 1916; Langley and Anderson, 1894–1895; and Cannon, 1929) and from previous experiments on denervation of the internal genitalia in the dog (Kuntz, 1919) and in the rodent (Rémy, 1886, Akutzu, 1903, and Bacq, 1931) it was considered sufficient for the purpose of the experiment to interrupt the hypogastric nerves or to extirpate the inferior mesenteric ganglion with pieces of the attached hypogastric nerves. In the former procedure, the operation could be performed unilaterally; in the latter, the denervation was necessarily bilateral. The technique was perfected until the operations could be performed within a very short time and with a minimum of trauma to the animal. The progress of the epididymal contents was followed by means of India ink injections as in the above group of animals.

The denervated animals numbered seventeen and can best be described in two groups. In the first, one-inch portions of the hypogastric nerves were removed from a series of eleven animals unilaterally only. The

TABLE 1

*A summary of the experimental animals**

ANIMAL NUMBER	WEIGHT	TIME OF DENERVATION RELATIVE TO TIME OF INJECTION	METHOD OF DENERVATION	DAYS FROM INJECTION TO AUTOPSY	POSITION OF INK AT AUTOPSY		REMARKS
					Right	Left	
	grams						
14	750	At same operation	Excision of one-inch portion of right hypogastric nerve	14	At third lobule of ant. portion of cauda epididymidis		
16	650	At same operation	Excision of one-inch portion of right hypogastric nerve	14	At beginning of second lobule of ant. portion of cauda epididymidis		
20	493	Seven days before	Excision of one-inch portion of right hypogastric nerve	17	At beginning of second lobule of ant. portion of cauda epididymidis		
29	576	One day after	Excision of one-inch portion of left hypogastric nerve	11		At middle of anterior portion of cauda epididymidis	
30	480	One day after	Excision of one-inch portion of right hypogastric nerve	13		Near end of anterior portion of cauda epididymidis	
31	506	One day after	Excision of one-inch portion of right hypogastric nerve	13	At very beginning of anterior portion of cauda epididymidis	At middle of posterior portion of cauda epididymidis	
32	457	One day after	Excision of one-inch portion of right hypogastric nerve	15	At beginning of second lobule of ant. portion of cauda epididymidis	Only two or three loops from beginning of ductus deferens	Right testis and epidid. larger than left. Both sides studied histologically
33	481	One day after	Excision of one-inch portion of right hypogastric nerve	15	At beginning of second lobule of ant. portion of cauda epididymidis	Only two or three loops from beginning of ductus deferens	
34	861	One day after	Excision of one-inch portion of right hypogastric nerve	14	At end of second lobule of anterior portion of cauda epididymidis	Only two or three loops from beginning of ductus deferens	
35	631	One day after	Excision of one-inch portion of right hypogastric nerve	14	At end of second lobule of anterior portion of cauda epididymidis	Only two or three loops from beginning of ductus deferens	
40	446	One day after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	16	Same as in no. 32		

* Cases in which the ink particles could not be definitely located are not recorded.

TABLE 1—*Concluded*

ANIMAL NUMBER	WEIGHT	TIME OF DENERVATION RELATIVE TO TIME OF INJECTION	METHOD OF DENERVATION	DAYS FROM INJECTION TO AUTOPSY	POSITION OF INK AT AUTOPSY		REMARKS
					Right	Left	
	grams						
46	438	One day after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	20	Between second and third lobules of anterior part of cauda epididymidis	Same as on right	
47	412	One day after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	20	In second lobule of ant. portion of cauda epididymidis		
48	518	One day after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	14	Same as in no. 31	Same as on right	Studied histologically
51	550	Three days after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	13		Near end of tubules of anterior portion of cauda epididymidis	Studied histologically
52	524	Three days after	Excision of inf. mesenteric ganglion and attached portions of hypogastric nerves	13	About two-thirds through the tubules of the ant. part of the cauda epidid.	Same as on right	Studied histologically
53	537	Three days after	Excision of one-inch portion of left hypogastric nerve	13	Four or five loops proximal to beginning of ductus deferens	Spread diffusely through proximal half of anterior portion of cauda epididymidis	Studied histologically. Masses of degenerating spermatozoa found unusually high in left cauda epididymidis

relationship between time of injection and time of denervation is given in table 1, which also shows the time elapsing between injection and autopsy.

The second group consisted of six animals in which the inferior mesenteric ganglion and attached portions of the hypogastric nerves were extirpated bilaterally. Details of these experiments are also recorded in table 1.

At autopsy, the position of the ink was determined grossly and with the help of a binocular dissecting microscope and immediately recorded on diagrams of the epididymis. The observations were made as rapidly as

possible with the animal under light ether anesthesia. Ligatures were placed at the ductus deferentes and the ductuli efferentes to minimize any possible movement of the epididymal contents due to asphyxia, post-mortem contraction, or fixation.

A review of the earlier literature reveals a prevalence of the idea that sympathetic denervation results in testicular degeneration (Ikeda, 1906; Kuntz, 1919; Takahashi, 1922; and Marconi, 1923). On the other hand, Bacq (1931) and Bacq, Brouha, and Desclin (1931) find no evidence of testicular degeneration following sympathectomy. In view of the conflicting opinions and in order to obtain information regarding the position of the India ink particles within the tubules of the epididymis, a histological study of the testis and its excretory ducts was made in five of the experimental animals. *In vitro* studies were made from some of the animals to observe any muscular activity in the epididymis and to study the condition of the spermatozoa contained within it. In the remainder of the animals, the testes and epididymides were fixed, dehydrated and cleared for further demonstration of the position of the injected ink. Photomicrographs were taken of one of these preparations (fig. 1) as well as of one of the stained sections of the epididymis (figs. 2 and 3).

Results. For convenience of description the cauda epididymidis is divided into two portions, an anterior and a posterior, which are very sharply demarcated one from the other. The anterior portion consists of a large number of lobules made up of the coiled ductus epididymidis which at this level is of comparatively very small caliber. The posterior portion is much shorter than the anterior and consists of coarse coils of tubules having relatively large caliber and finally merging imperceptibly with the straight ductus deferens. An excellent diagram showing these relationships has been published by Young (1931).

Data obtained from injections in the first group of five normal animals indicate that the ink particles pass through the ductus epididymidis in about sixteen days. In the three animals autopsied after fourteen days, the particles were concentrated in the first few coils of the posterior part of the epididymis. In the animal autopsied after sixteen days, the ink particles were found just proximal to the ductus deferens. In the fifth animal, at the time of the exploratory operation on the fourteenth day, the ink particles were found in the second and third loops of the posterior portion of the tail of the epididymis; and at the time of autopsy, on the nineteenth day, they were found concentrated at the very distal end of the ductus deferens. These results agree essentially with those of Toothill and Young (1931) who in a much larger series of animals found that India ink particles passed through the epididymis in from fourteen to eighteen days.

The data from the denervated animals are summarized in the table

on pages 584–585, but a general statement of the results may be made at this point. In every case there was a marked enlargement of the seminal vesicles either unilaterally or bilaterally depending upon the denervation, a phenomenon also observed by Rémy (1886) and Bacq (1931). Especially striking were the results obtained from the unilaterally denervated animals. On the denervated side, after 13 to 20 days, the ink particles were found to have reached no further than the first to fourth lobule of the anterior portion of the tail of the epididymis, amounting to less than a fifth of the length of this portion of the duct (fig. 1, animal 14). Two animals, nos. 53 and 29, constitute exceptions. In the former, the ink was found spread diffusely through the duct of the epididymis somewhat beyond the proximal half of the anterior portion of the tail. In the latter,

Fig. 1. Lower pole of testis and epididymis (animal 14) under low magnification showing ink in proximal portion of anterior part of cauda epididymidis.

the ink was found near the middle of the anterior portion of the cauda epididymidis, but not diffusely spread.

On the normal side, however, the ink particles were with only one exception found two to five loops proximal to the beginning of the ductus deferens, as can be seen from figure 2 which shows the ink in one of the very last loops of the posterior part of the cauda epididymidis (animal 32). In no. 30, the ink had reached only to the end of the anterior portion of the tail of the epididymis.

In the group of animals which were denervated bilaterally, the ink was found in the first two or three lobules of the anterior portion of the tail of the epididymis, except in the two animals which were denervated on the third day after the injection. In these, the ink had progressed through a good part of the anterior portion of the tail of the epididymis. The interval between injection and denervation undoubtedly played a part here.

Some of the observations made on excised portions of the epididymides in mammalian Ringer solution are of interest. A vigorous muscular activity was observed in the tubule under the compound microscope. The activity, however, was not a true peristalsis, but resembled a segmentation (Cannon, 1902). The contents of the duct were seen to be driven to and fro but with no apparent progress in either direction. With reference to the motility of spermatozoa within the ductus epididymidis, a question which has been reviewed by Young (1929 and 1931), it was noted that spermatozoa within the excised tubules were non-motile, but they became motile as soon as they escaped into the solution from the torn end of the tubule. The observations were not sufficient in number to permit a defi-

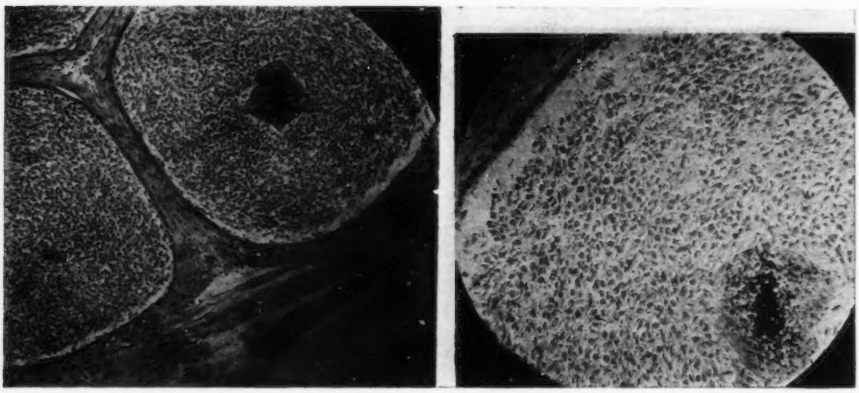


Fig. 2 Fig. 3

Fig. 2. Photomicrograph of section through tail of epididymis showing ink in one of very last loops of cauda epididymidis (animal 32).

Fig. 3. Photomicrograph of same section as in figure 2 seen under high power and showing ink within a mass of degenerating spermatozoa.

nite conclusion, but they point toward the idea that spermatozoa are moved passively through the epididymis.

The histological study showed no abnormal changes in either the testis or its excretory ducts in the sympathectomized animals. The germinal epithelium showed active spermatogenesis; the epithelium of the excretory ducts showed normal secretory activity, and a few mitotic figures were found as in the normal epididymis. There was no evidence of India ink particles within the cells lining the tubules of the tail of the epididymis which has been reported by Richard (1929). In animal 32 (figs. 2 and 3), the ink particles were found in the masses of degenerating spermatozoa which are normally present in the distal end of the epididymis (Simeone

and Young, 1931). In one instance, these degenerating masses were found as high as at the level of the first few coils of the posterior portion of the tail of the epididymis. This in itself would indicate a stagnation of the contents of the epididymis.

Discussion. The results reported above demonstrate the presence of a neuromuscular mechanism in the ductus epididymidis as one of the factors responsible for the distal progress of the epididymal contents. The mechanism is impaired by sympathetic denervation. The value of the injection method for following the progress of the epididymal contents has been discussed by Toothill and Young (1931). I may merely add here that the injections gave rise to no abnormal processes in the epididymis. The ink particles were incorporated in some cases with the masses of degenerating spermatozoa which are normally found in the distal portion of the cauda epididymidis. Evidence has also been presented that the spermatozoa are probably carried passively through the duct so that the rate of passage of these cells and that of the ink particles cannot differ materially.

The experiments, however, have not definitely revealed the nature of the change produced by the denervation. It is certain that denervation does not produce complete paralysis of the ductus epididymidis, since powerful segmentation was observed in excised portions of the duct. Similar rhythmic contractions were observed in the excised ductus deferens by Waddell (1917). It seems possible that the denervation produced a change from normal peristaltic activity to one of mere segmentation. This would be analogous to the modified activity observed in the intestine (Thomas and Kuntz, 1926) after paralysis of nervous control. In the intestine, however, function returns after a brief preliminary paralysis (Cannon, 1906). The above experiments indicate that the impairment of function persists for at least twenty days.

The importance of the factors responsible for the distal progress of spermatozoa through the ductus epididymidis becomes evident in view of the recently developed ideas regarding the epididymal spermatozoön relationship (Young, 1929 and 1931; Simeone and Young, 1931). During the time spent by spermatozoa in traversing the long coiled duct of the epididymis some nine feet in length, these cells are at one time undergoing a ripening process and at another a process of degeneration. Spermatozoa which are either too young or too old are incapable of effecting fertilization. It would be interesting in this connection to test the fertilizing capacity of spermatozoa from sympathectomized males by means of artificial insemination.

Bacq (1931) has demonstrated impotency of the male rodent following sympathetic denervation of the genital organs. He attributed the impotency to failure of contraction on the part of the ductus deferentes and

seminal vesicles, thus making ejaculation impossible, and also to lack of prostatic secretion. Whereas the absence of ejaculation is of direct importance for the reproductive capacity of the male, the prostatic secretion is of minor significance in view of the recent experiments involving artificial insemination in the guinea pig (Young, 1931; Young and Simeone, 1930). On the other hand, stagnation of the contents of the epididymis after sympathetic denervation would be very important since additional aging of the spermatozoa within the ductus epididymidis might result in a loss of their fertilizing capacity.

A final point should be mentioned for brief discussion at this time. Recent work on vasectomy, in contrast to earlier views, indicates that obstruction of the ductus deferens does not lead to degeneration of testicular parenchyma, as would be the case in obstruction of the ducts of the kidney or pancreas. Moore and Quick (1924) suggest that the testicular products are swept outward and away from the testis by a muscular activity on the part of the epididymis. Pressure atrophy of the testis is thus prevented. Nonidez (1924), however, suggests that extensive atrophy is prevented by resorption of testicular products through tubules in which the germinal epithelium has undergone a pressure atrophy. Experiments are being planned for the investigation of this problem.

CONCLUSIONS

1. A neuromuscular mechanism in the ductus epididymidis is one of the factors which by its peristaltic activity is responsible for the distal progress of the contents of the epididymis.

2. The mechanism is impaired by sympathetic denervation of the internal genitalia.

3. The significance of these facts for the physiology of reproduction in the male guinea pig is discussed.

It is my pleasure to thank Doctor Cannon and Doctor Gregersen for the kind interest which they have had in this work.

BIBLIOGRAPHY

Akutzu, S. 1903. Pflüger's Arch., xcvi, 541.
Bacq, Z. M. 1931. This Journal, xcvi, 321.
Bacq, Z. M., L. Brouha and L. Desclin. 1931. Compt. rend. soc. biol., cviii, 1169.
Benoit, J. 1926. Arch. d'anat., d'histol., et d'embryol., v, 175.
Cannon, W. B. 1906. This Journal, xvii, 429.
1902. This Journal, vi, 251.
1929. Bodily changes in pain, hunger, fear, and rage. New York.
Gaskell, W. H. 1916. The involuntary nervous system. London and New York.
Ikeda, R. 1906. Zentralbl. f. Physiol., xx, 590.
Kuntz, A. 1919. Anat. Rec., xvii, 203.
Langley, J. N. and H. K. Anderson. 1894–5. Journ. Physiol., xvii, 177. 1895. Journ. Physiol., xix, 122.

MARCONI, P. 1923. Compt. rend. soc. biol., lxxxviii, 356.
MOORE, C. R. AND W. J. QUICK. 1924. Amer. Journ. Anat., xxxiv, 317.
NONIDEZ, J. F. 1924. Amer. Journ. Anat., xxxiv, 393.
OSLUND, R. M. 1928. Journ. Amer. Med. Assoc., xc, 829.
RÉMY, C. 1886. Journ. d. l'Anat. et Physiol., xxii, 205.
RICHARD, M. 1929. Schweiz. Med. Wochenschr., x, 1269.
SIMEONE, F. A. AND W. C. YOUNG. 1931. Journ. Exper. Biol., viii, 163.
TAKAHASHI, N. 1922. Pflüger's Arch., cxciii, 322.
THOMAS, J. E. AND A. KUNTZ. 1926. This Journal, lxxvi, 606.
TOOTHILL, M. C. AND W. C. YOUNG. 1931. Anat. Rec., l, 95.
WADDELL, J. A. 1917. Journ. Pharm. Exper. Therap., viii, 551.
YOUNG, W. C. 1929. Journ. Morph. and Physiol., xlvii, 479.
1931. Journ. Exper. Biol., viii, 151.
YOUNG, W. C. AND F. A. SIMEONE. 1930. Proc. Soc. Exper. Biol. and Med., xxvii, 838.

OBSERVATIONS ON THE FLOW OF BLOOD IN THE FEMORAL ARTERY OF THE DOG EIGHT TO THIRTY-FOUR MONTHS AFTER LUMBAR SYMPATHECTOMY

J. F. HERRICK, HIRAM E. ESSEX AND EDWARD J. BALDES

From the Division of Physics and Biophysical Research and the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota

Received for publication November 25, 1932

In a previous paper we reported the effect of lumbar sympathectomy on the flow of blood in the femoral artery of the dog under local and ether anesthesia. Under local anesthesia the flow of blood on the sympathectomized side was found to be at least twice that on the normal side. Under ether anesthesia there is not an appreciable difference in the flow of blood of the two sides, since this anesthetic abolishes the vasomotor tonus.

The flow of blood was measured by the Thermo-Stromuhr method developed by Rein, and slightly modified by Herrick and Baldes. The principle of this method is as follows: at a definite place on the wall of the vessel, two small, platinum electrodes, placed diametrically opposite, lead an electric current through the blood flowing in the vessel in situ. This produces a small quantity of heat in the blood and the resulting change in temperature is noted by means of a sensitive differential thermocouple. The more slowly the blood flows, the greater will be this resulting change in temperature. A high-frequency alternating current is used, and the only effect of this current is that of heating.

It should be of importance to determine experimentally whether in the absence of the lumbar sympathetic nerves, the vessels of the limb regain their tonus sufficiently to decrease the flow of blood wholly or in part to the level obtained before sympathectomy. Since sympathectomy is being employed for relief of certain pathologic conditions such as Raynaud's disease, in which the peripheral circulation is inadequate, such experiments should indicate whether sympathectomy may be expected to give permanent relief in cases in which it is used (Brown and Adson, 1925, 1929).

In an attempt to answer this question the flow of blood in the femoral artery was again measured eight to twelve months following sympathectomy. Four of the original series of dogs were used. As in previous experiments the operative procedures were carried out under local anesthesia. In addition, measurements were made on two animals not included in the original series, but on which sympathectomy had been performed for a different purpose, respectively, nineteen and thirty-four months previously.

In none of the animals in which the flow of blood had been measured previously was there any indication of return to the level that had existed before sympathectomy (table 1). Except in one animal the flow of blood was as great as, or greater than, it had been just after sympathectomy, but in every instance the flow in the femoral artery on the sympathectomized side was at least twice that on the normal side. In the two animals in which the flow of blood had not been measured previously, the animal on which sympathectomy had been done nineteen months before had almost exactly twice the normal flow in the femoral artery on the side of operation. The other animal, on which sympathectomy had been done thirty-four

TABLE 1

Comparative flow of blood in the femoral arteries of dogs following lumbar sympathectomy on the left side (with the exception of dog 6)

DOG	OBSERVATION	WEIGHT	FLOW EACH MINUTE		RATIO
			Left femoral artery	Right femoral artery	
		kgm.	*cc.*	*cc.*	
1	First observation	17.8	201	84	2.39
	Eight months later	17.6	236	108	2.18
2	First observation	13.5	297	99	3.00
	Eleven months later	17.8	377	188	2.00
3	First observation	13.2	294	125	2.35
	One year later	16.0	242	90	2.69
4	First observation	13.7	105	54	1.94
	One year later	15.1	271	130	2.08
5	Nineteen months after sympathectomy	9.5	250	124	2.02
6	Two years ten months after sympathectomy (three ganglia on right side; one ganglion on left side)	8.1	69	112	1.62

months previously, had three ganglia removed from the right lumbar chain and one from the left. The flow of blood in the right femoral artery was 1.62 times that in the left femoral artery. In spite of the incomplete operation and the lapse of nearly three years, the flow of blood in the two femoral arteries differed significantly.

SUMMARY AND CONCLUSION

The flow of blood in the femoral artery was measured eight to twelve months after lumbar sympathectomy in four of a series of animals in which measurements previously had been made under similar conditions. The flow on the side of operation was still twice that on the intact side in every

animal. Measurements on two other animals, on which sympathectomy had been done nineteen and thirty-four months previously, gave comparable results. It therefore seems reasonable to conclude that the alterations in flow of blood of the femoral artery of the dog following lumbar sympathectomy are apparently permanent.

BIBLIOGRAPHY

BROWN, G. E. AND A. W. ADSON. 1925. Amer. Journ. Med. Sci., clxx, 232.
1929. Arch. Neurol. and Psychiat., xxii, 322.
HERRICK, J. F. AND E. J. BALDES. 1931. Physics, i, 407.
HERRICK, J. F., H. E. ESSEX AND E. J. BALDES. 1932. This Journal, ci, 213.
REIN, H. 1928. Zeitschr. f. Biol., lxxxvii, 394.

THE CAUSE OF MAMMARY DEVELOPMENT DURING PSEUDO-PREGNANCY IN THE RABBIT

S. A. ASDELL AND G. W. SALISBURY

From the Laboratory of Animal Nutrition, State College of Agriculture, Cornell University, Ithaca, N. Y.

Received for publication November 10, 1932

Recent work on the causes of mammary development has tended to divert attention from the reproductive tract to the anterior pituitary. Corner (1930) and Asdell (1931) have shown that alkaline extracts of anterior pituitary injected into the spayed virgin rabbit produce a development and secretion of the mammary gland almost, if not quite, equal to that which is observed at the end of pregnancy. Riddle, Bates and Dykshorn (1932) by the use of an acid extract have also produced mammary secretion. As the latter workers do not report development and as their active substance appears to be much more stable than that investigated by Corner, the identity of the two substances cannot be assumed.

In order to determine the effects of the various hormones of the reproductive tract, Turner and Gardner (1931) have tested the mammary stimulating effects of oestrin and corpus luteum extracts on the rabbit. They find that oestrin alone produces no development in rabbits which have previously been pregnant or pseudopregnant. As Turner and Frank (1930) have shown that oestrin produces duct development and slight milk secretion in the rabbit spayed before puberty, these experiments suggests that oestrin has an effect solely on duct growth and is not concerned with alveolar development, i.e., with the part of the gland which is necessary for secretion in quantity. Higher doses of oestrin seemed to produce no further development than moderate doses. These results lead to the inference that oestrin itself will only produce the degree of mammary development which is normally observed in the rabbit ready for mating. By injecting considerable amounts of oestrin together with a corpus luteum extract (method of preparation not given) they obtained much greater development and milk secretion, but the development fell short of that which followed anterior pituitary injections. The rabbits employed had been ovariectomised and the injections in one were made immediately following a period of pseudopregnancy and in the other after involution had set in. There is no doubt that the injections have prevented retrogression in the first case, and have produced reactivation in the second.

With alkaline extracts of anterior pituitary similar to those used by other workers, mammary growth and secretion were obtained equal to that normally found during the first few days after parturition. Turner and Gardner conclude that the anterior lobe hormone acts only on the secretory cells stimulating them to activity and that it does not produce an increase in the number of alveoli. We are unable to agree with this conclusion as virgin rabbits which have been injected with anterior lobe extract after spaying show a full pregnant development although they have previously experienced no further growth than that produced by their own puberty producing substances.

It is not clear, as Turner and Gardner point out, whether the oestrin and corpus luteum extracts act directly on the mammary gland or whether they stimulate the anterior lobe to activity; this can only be determined by injections made into the hypophysectomised animal. Further, although various organ extracts injected in large quantities may produce certain results, it does not follow that these results are produced in the normal animal by the same mechanism though the probability is strong that the factors under investigation are involved. This reservation is especially true in the case of mammary growth where a variety of substances is known which produce mammary growth or secretion. Also, one of these substances, oestrin, is believed to be produced both in the ovary, and, during pregnancy, by extra ovarian agencies. With the view of finding, if possible, the stimuli which act in the normal animal the experiments described in this paper have been devised.

The rabbit, with pseudo-pregnancy and lactation after a sterile coitus, clearly rules out, in this species at least, the possibility of the intervention of the fetus and adnexa in the mammary development associated with these phenomena. But the question arises whether coitus acts directly on the anterior lobe causing the hormone producing mammary development to be secreted, or whether the stimulus arises in the reproductive tract. Corner (1930) points out that progestin injections in the absence of pregnancy have no effect on the mammary gland. He quotes Jares (unpublished work) who finds that corpora lutea produced by the injection of human pregnant urine are without effect on the mammary gland. He states, however, that the examination of the mammary glands was made at the end of thirty-three days when three successive sets of corpora lutea had been produced by the injections, so that the possibility arose that involution had set in. This seems unlikely as we might expect the new corpora lutea at least to maintain any development which had occurred. But when pregnancy was maintained in the absence of the ovaries with the aid of progestin full mammary development occurred. The evidence at Corner's command led to the inference that mammary development in the pregnant rabbit is directly due to the anterior lobe, the ovary having

no influence except by maintaining pregnancy. If these inferences be correct it is logical to believe that the pseudo-pregnant development also has an extra ovarian origin. It has been the object of our work to test this hypothesis and our results fail to bear it out.

EXPERIMENTAL. 1. In order to decide whether the act of coitus has a direct influence on the mammary stimulating mechanism of the anterior lobe similar to its effect on the ovulation producing mechanism, two virgin does were mated with a vasectomised buck, one full copulation being allowed to each. Six days later the ovaries were removed under ether anesthesia. In each case ovulation had occurred and fresh pink corpora lutea were present. At the thirteenth day after coitus the rabbits were killed and the state of the mammary glands observed. In each case some duct development had occurred but no alveolar development or thickening of the glands. It was evident that mammary growth had ceased with the removal of the ovaries. The gland areas were quite white and anemic without any sign of the increased vascularity of pseudo-pregnancy. Here we may state that the colour of the mammary area is a very good criterion of the activity of the gland, a growing gland is vascular and pink while an involuting gland is white and anemic. In a secreting gland the ducts are obviously swollen with milk. It is evident from the results of this experiment that the ovary is a necessary link in the chain of events leading to mammary development. The action is not directly on the anterior pituitary.

2. Further experiments were made to determine whether mammary development may take place in the absence of coitus. It is to be expected that such may occur as occasionally pseudo-pregnancy with mammary development takes place in isolated doe rabbits.

Luteinisation of follicles was produced by the subcutaneous injection of a gonad stimulating extract of anterior pituitary for three or four days. The rabbits were killed thirteen days after injections ceased and the ovaries and mammary glands were examined. Three rabbits were used, in each case luteinisation had occurred and in two at least ovulation had evidently occurred as some of the corpora lutea had rupture points. All three mammary glands were well developed and thickened and all had milk in their ducts. In one case retrogression was beginning to set in and the corpora lutea of this rabbit were also beginning to lose their blood supply. Probably ovulation had occurred after the first injection.

As controls three rabbits which had previously borne young were ovariectomised and subsequently injected with the same extract under the same conditions. On killing these at the thirteenth day after the cessation of injections their mammary glands were found to be thin and atrophic. Very little, if any, glandular development had occurred as a result of the injections, but a small quantity of milk was present in the ducts. A slight

reddening of part of the mammary area had occurred in one of the rabbits but as this rabbit showed some infection in this area, the increased vascularity was put down to the infection. In no case was there a development similar to that found in the experimental group.

It was concluded that luteinisation of the ovary is effective in bringing about mammary development equal to that found at the end of pseudo-pregnancy.

3. Six rabbits were injected intravenously with 7 to 10 cc. of pregnant human urine. Twenty-four hours or less later laparotomy was performed. In all cases ovulation had occurred. The mammary glands were examined and found to be undeveloped. One of these animals was killed at seven and a half days after the injection. Mammary development was proceeding. The other five were killed from the eleventh and a half day to the fifteenth day. In the eleventh and a half day rabbit the mammary development, while evidently in progress, appeared to be rather less than is usual at this stage. The corpora lutea were healthy looking. In the other four rabbits full pseudo-pregnant development had occurred at fourteen and fifteen days and milk was being secreted. It is evident that the artificial production of corpora lutea is accompanied by the usual pseudo-pregnant changes. It may be mentioned that the uterine changes characteristic of pseudo-pregnancy were occurring in all these rabbits.

Discussion. It is evident from these experiments that the mammary development of pseudo-pregnancy in the rabbit is related to the activity of the ovaries. Removal of these organs after the initiation of pseudo-pregnancy effectively checks development. Evidence is given by Asdell and Hammond (1933) that a similar growth occurs in the hysterectomised pseudo-pregnant rabbit so that the uterus is not a factor. Whether the ovary acts directly on the mammary gland or through the anterior lobe our experiments fail to show. Ablation of the pituitary during pseudo-pregnancy, if it prevented mammary development, would be inconclusive as such a result might be due to the removal of a gonad stimulating substance rather than to the removal of a direct mammary stimulating substance.

The experiments on the production of lutein tissue show that the act of coitus is not the necessary stimulus to mammary activity. The possibility that ovulation itself provides the stimulus has not been entirely ruled out but we do not think it has any effect as it seems to be a mechanical result of the action of gonad stimulating factors. One of us (S. A. A.) has found that no mammary development follows the pricking of ripe follicles. No corpora lutea are formed under these circumstances, a fact which has been also reported by Walton and Hammond (1928). The presence of lutein tissue, the result of all three methods of stimulation, coitus, anterior pituitary, prolan, is apparently the determining factor. This brings us

back, for pseudo-pregnancy at least, to the theory of Ancel and Bouin (1911) and of Hammond and Marshall (1914) that the corpus luteum is an essential element in mammary stimulation. We think this factor is also at work at any rate in the first half of pregnancy in the rabbit as conditions at this time seem to be similar to those which we have been discussing. There is evidence that other factors are involved in the second half of pregnancy. These are discussed elsewhere (Asdell and Hammond, 1933).

CONCLUSIONS

1. The stimulus for mammary development during pseudo-pregnancy in the rabbit originates in the ovary.

2. This stimulus is associated with the corpus luteum and is independent of coitus and probably of ovulation except as these phenomena produce corpora lutea.

3. It is believed that conditions in the first half of pregnancy are the same as during pseudo-pregnancy.

BIBLIOGRAPHY

ANCEL, P. AND P. BOUIN. 1911. Journ. de Physiol. et de Path. Gen., xiii, 31.
ASDELL, S. A. 1931. Cornell Veterinarian, xxi, 147.
ASDELL, S. A. AND J. HAMMOND. 1933. This Journal, ciii, 600.
CORNER, G. W. 1930. This Journal, xcv, 43.
HAMMOND, J. AND F. H. A. MARSHALL. 1914. Proc. Roy. Soc., B. xxxvii, 422.
RIDDLE, O., R. W. BATES AND S. W. DYKSHORN. 1932. Proc. Soc. Exp. Biol. and Med., xxix, 1211.
TURNER, C. W. AND A. H. FRANK. 1930. Mo. Agric. Expt. Sta., Res. Bull. 145.
TURNER, C. W. AND W. U. GARDNER. 1931. Mo. Agric. Expt. Sta., Res. Bull. 158.
WALTON, A. AND J. HAMMOND. 1928. Brit. Journ. Exp. Biol., vi, 1900.

THE EFFECTS OF PROLONGING THE LIFE OF THE CORPUS LUTEUM IN THE RABBIT BY HYSTERECTOMY

S. A. ASDELL AND J. HAMMOND

From the Laboratory of Animal Nutrition, State College of Agriculture, Cornell University, Ithaca, N. Y., and Institute of Animal Nutrition, School of Agriculture, Cambridge University, England

Received for publication November 10, 1932

Although much work during the last few years has directed attention to the anterior pituitary as the source of the stimulus for mammary growth, recent experiments by Asdell and Salisbury (1933) have shown that in the pseudo-pregnant rabbit the effective stimulus, whatever it may be, is not released unless corpora lutea are present. The literature on the subject is discussed in that paper. In view of this result the possibility arises that the further development seen during pregnancy in this species is due to the prolongation of the life of the corpus luteum which pregnancy entails. Corner (1930), however, found that full pregnant development occurs in the spayed doe when pregnancy is maintained by the use of progestin, although this substance in the absence of pregnancy has no such effect. If a means can be found by which the life of the rabbit's own corpora lutea may be prolonged, without the complicating factor of pregnancy, some light might be thrown on the problem. Such a means is at our disposal.

L. Loeb (1923) found that hysterectomy in the guinea pig prolongs the life of the corpus luteum. He obtained a persistence in one case for as long as sixty days instead of the usual sixteen days. This was accompanied by mammary proliferation and milk secretion. We find that this operation is followed by the prolongation of the life of the corpus luteum in the rabbit, but the increased duration is shorter than that found by Loeb in the guinea pig. This prolongation is not merely one of form, but of function, in part at least, as ovulation does not occur after coitus in rabbits with prolonged corpora lutea.

EXPERIMENTAL. The uteri together with the cervices and in some cases the Fallopian tubes of a number of does were removed before puberty. No difference in subsequent behaviour was observed whether the uteri or uteri and tubes were removed, so this detail has been ignored in subsequent discussion. At a uniform age, after puberty, the does were mated. They were killed at twenty-four, twenty-five and twenty-nine days after coitus. The normal duration of pseudo-pregnancy is sixteen days after

which the corpora lutea, uteri and mammary glands rapidly regress. The appearance and measurements of the corpora lutea together with the weight of the mammary glands in the experimental animals were taken and compared with similar observations for normal pregnant and pseudo-pregnant does. The day before killing an opportunity was given for each rabbit to mate with a buck. The effect of this mating, if it occurred, on the ovaries was noted.

The mammary glands were prepared for weighing in this way. The skin was removed from the rabbits by flank incisions, care being taken not to cut the glands in the thoracic and inguinal regions. It was then stretched and pinned out, hair downwards. The gland area was flooded with 95 per cent alcohol and any adherent muscle and fat were removed. The entire mammary area including its muscle, was then cut around with a sharp scalpel and flayed off the skin. The nipples were released by a close incision round each. In the pregnant rabbits the area removed nearly corresponded to that of the glands, but in the cases where mammary development was not so great an area of muscle as equal as possible to that of the pregnant rabbits was removed. All the rabbits were about the same weight.

Results. Pregnant rabbits, 24 days after coitus; 4 cases. Corpora lutea present in all cases, noted as large in 3, large (?) in 1. Weights of mammary glands 62, 48, 72, 63. Average 61 grams. Mammary glands in all cases thick. One copulated, no ovulation.

Pseudo-pregnant rabbits, 24 days after coitus; 4 cases. All corpora lutea small. Weights of mammary glands 39, 37, 34, 32. Average 36 grams. Mammary glands small and atrophic, milk in one. Three copulated and ovulated.

Hysterectomised rabbits, 24 days after coitus, 4 cases. Corpora lutea large in 3 cases, small in 1. Weights of mammary glands 40, 41, 39, 46. Average 41 grams. Mammary glands variable, 2 small, 1 reddish, thick, no milk, 1 reddish, not thick. All copulated but only one of the four ovulated.

Hysterectomised rabbits, 25 days after coitus, 3 cases. Corpora lutea large. Weights of mammary glands 37, 49, 41. Average 42 grams. All mammary glands thin but reddish. No ovulations after coitus.

Pregnant rabbits, 29 days after coitus, 5 cases. Corpora lutea large. Weights of mammary glands 47, 84, 76, 92, 104. Average 81 grams. Mammary glands thick in all cases. Two copulated but did not ovulate.

Pseudo-pregnant rabbits, 29 days after coitus, 5 cases. Corpora lutea moderate to very small. Weights of mammary glands 31, 26, 21, 33, 35. Average 29 grams. Mammary glands atrophic in all cases. Ovulation occurred after coitus in all cases.

Hysterectomised rabbits, 29 days after coitus, 9 cases. Corpora lutea moderate to small. Weights of mammary glands 31, 42, 29, 27, 38, 19, 27, 28, 28. Average 30 grams. Mammary glands all atrophic. Ovulation occurred in the 8 which copulated.

Measurements of corpora lutea were made in the largest free hand sections from two corpora lutea in each animal after fixation in 10 per cent formalin. The measurements were taken at right angles. The averages

of these measurements and the average mammary gland weights are given in table 1.

It will be seen that the corpora lutea of pregnancy maintain their size at the 29th day while the pseudo-pregnant corpora lutea have already decreased by the 24th day, there is only a slight difference in our measurements following this state between the 24th and 29th days' corpora. The decrease in size is abrupt after the failure of the corpora lutea, but following this the process of reabsorption is slow. The initial decrease may be connected with a sudden reduction in the blood supply. The difference in vascularity between a functioning and a non-functioning corpus luteum is striking in the rabbit. But the corpora lutea of the hysterectomised rabbits at 24 and 25 days are much larger than the pseudo-pregnant ones, approaching those of the pregnant corpora. At 29 days regression has set in in all cases although the hysterectomised are still appreciably larger

TABLE 1

Measurements of corpora lutea and weights of mammary glands

(Measurements on micrometer scale, 56 = $\frac{1}{8}$ inch)

TYPE OF RABBIT	NUMBER OF RABBITS	MEASUREMENTS OF CORPORA LUTEA—AVERAGE DIAMETERS	WEIGHTS OF MAMMARY GLANDS—AVERAGE WEIGHTS
			grams
Pregnant, 24 days	4	53.5	61.2
Pseudo-P., 24 days	4	35.2	35.5
Hysterectomy, 24 days	4	44.0	41.5
Hysterectomy, 25 days	4	49.4	42.3
Pregnant, 29 days	5	57.2	80.6
Pseudo-P., 29 days	5	30.2	29.2
Hysterectomy, 29 days	9	36.8	29.9

than the normal pseudo-pregnant. The mating trials show that the size of the corpus luteum is directly related to its function. Ovulation did not occur after coitus in any of the pregnant rabbits while it invariably ensued in the pseudo-pregnant does both at 24 and 29 days. The effectiveness of these corpora lutea as inhibitors of follicular development had ceased. In the hysterectomised rabbits at 24 and 25 days, ovulation followed coitus in 1 out of 7 cases. In the one case where ovulation occurred the corpora lutea were recorded as small, this being confirmed by measurement. At 29 days all copulations were followed by ovulation. It is clear that copulation may occur whatever the condition of the corpus luteum, but ovulation only follows if the corpus luteum is being absorbed.

The mammary glands of the pregnant rabbits weighed more at 29 days (81 grams) than at 24 days (61 grams), while the pseudo-pregnant glands during this time were shrinking (36 grams at 24 days and 29 grams at 29

days). At 24 and 25 days these glands in the hysterectomised rabbits were a little larger (42 grams) than the corresponding pseudo-pregnant glands but they were not larger than the usual 16 days pseudo-pregnant glands. We judged that their development had ceased at that stage and that they were then being maintained at that level. At 29 days when the corpora lutea were no longer active as ovulation suppressors, the mammary glands have been definitely reduced in size (30 grams). In appearance they were pale and anemic.

Discussion. These results show that following hysterectomy, in the rabbit as in the guinea pig, the life of the corpus luteum is prolonged. This is shown not only by measurements but also by the suppression of ovulation after coitus. In these rabbits no ripe follicles were present. As ripening of follicles and ovulation (if coitus is allowed) occur immediately after the end of pregnancy when the corpora lutea are regressing, we consider that the physiological findings, taken in conjunction with the size of the corpora lutea, denote a functional persistence for between 24 and 29 days after the initial copulation. This is in place of the usual 16 days which is found in pseudo-pregnancy and 30 to 32 days in pregnancy. Although this is less than the time reported by Loeb for the guinea pig, it is noteworthy and requires explanation.

The prolongation of the life of the corpus luteum during pregnancy seems to be due to the presence of the uterus and its contents, and, as metritis in the cow is often associated with a persistent corpus luteum, this persistence appears to be the result of the action of a uterine factor on the one hand. On the other hand, Smith and White (1931) have shown that after hypophysectomy in the mated rabbit the corpora lutea regress. Heavy lactation leads to their absorption in the rabbit, also possibly by removal of anterior pituitary substance from the blood (Hammond and Marshall, 1925). Removal of the corpora lutea, as is well known, in the majority of species including the rabbit is followed by cessation of pregnancy. There must be a series of reciprocal relations between the activities of the uterus, corpus luteum and hypophysis which are not easy to unravel. We suggest that the persistence in the hysterectomised rabbits may be due to a sparing effect on the hypophyseal secretion. The uterus may use a certain quantity of some substance that is required by the corpus luteum for its proper functioning. If then, no uterus is present to use it, more may be spared for the corpora lutea which may be able to persist as a consequence. During pregnancy the evidence points to an increased secretion by the hypophysis associated with its hypertrophy at this time or to the formation of an hypophysis-like substance by the placenta. But the relations are at present too involved to allow us to do more than indicate a possible explanation. The theory is logical since most, if not all, the functions ascribed to the anterior lobe are essentially

growth phenomena so that the uterus may require its share of the hypophyseal secretion.

Although the corpora lutea are functioning as inhibitors of follicular ripening, yet no more mammary growth has taken place after hysterectomy than is normal during pseudo-pregnancy. This might suggest that mammary development may not be concerned with the corpora lutea at all; but Asdell and Salisbury (1933) have produced evidence that the mammary development of pseudo-pregnancy is due to an ovarian factor which is the corpus luteum. Accepting this as correct, we may infer that the corpora lutea alone cannot carry mammary development further than the pseudo-pregnant stage. An alternative is that the corpora lutea in the hysterectomised group lose their function as mammary growth inducers although their ovulation inhibiting function is unimpaired. But Corner's (1930) significant observation that progestin alone is without effect on the mammary gland, while progestin plus a pregnancy maintained by its action is instrumental in bringing about full mammary development strongly suggests that mammary development under these conditions is due to an extra ovarian stimulus. This evidence suggests that the first theory, that the corpora lutea cannot carry the mammary development further, is the correct one although it does not eliminate the alternative. Parkes (1929) produced a new set of corpora lutea toward the end of a normal pseudo-pregnancy by injections of anterior pituitary extract. The injections were continued for 10 to 18 days and control injections into ovariectomised rabbits showed that the extracts themselves were without effect on the mammary gland. In the experimental animals, as a result of the induced corpora lutea (24 to 32 days under total corpus luteum influence) mammary development equal to that of pregnancy resulted. The plates accompanying the paper indicate that some alveolar development has taken place, but the weights of the mammary glands are, except in two cases, within the range of pseudo-pregnant development. In one of the two cases the gland weight, 95 grams, is easily that for a pregnant rabbit. The fact that in our experiments at 24 days the pregnant mammary gland (61 grams) weighs so much more than it does in the hysterectomised animals (41 grams) in which the corpora lutea had persisted, supports the view that the stimulus for mammary development further than it is carried in pseudo-pregnancy is extra ovarian, and the balance of the evidence is decidedly in favour of this view.

CONCLUSIONS

1. The corpora lutea in the mated hysterectomised rabbit retain their size and their ovulation inhibiting function for between 24 and 29 days, representing a life of approximately 10 days longer than that of the pseudo-pregnant corpus luteum, but about 6 days shorter than that of the corpus luteum of pregnancy.

2. This prolongation of life is unaccompanied by further development of the mammary gland which is maintained at the pseudo-pregnant level.

3. The bearing of (2) on the complete mammary development of pregnancy is discussed; the balance of the evidence is in favour of an extra-ovarian origin for the stimulus which is effective during the latter half of pregnancy.

BIBLIOGRAPHY

ASDELL, S. A. AND G. W. SALISBURY. 1933. This Journal, ciii, 595.
CORNER, G. W. 1930. This Journal, xcv, 43.
HAMMOND, J. AND F. H. A. MARSHALL. 1925. Reproduction in the rabbit. Edinburgh.
LOEB, L. 1923. Proc. Soc. Exp. Biol. and Med., xx, 441.
PARKES, A. S. 1929. Proc. Roy. Soc., B. civ, 189.
SMITH, P. AND W. E. WHITE. 1931. Journ. Amer. Med. Assoc., xcvii, 1861.

ANIMAL ADAPTATION TO ENVIRONMENTAL TEMPERATURE CONDITIONS[1]

CORDELIA OGLE AND C. A. MILLS

From the Laboratory for Experimental Medicine, University of Cincinnati

Received for publication October 29, 1932

In our study of climatic effects we have become convinced that the temperature factor is a very important one in determining the stimulating quality of man's environment. The mean monthly temperature level and the variability of daily temperatures across the mean possess greatest weight. Most stimulating for man seems to be a mean temperature between 38°F. and 64°F., with frequent fluctuations of moderate extent across this mean range. Various phases of human social and economic development have proceeded with greatest rapidity and been most extensive in those regions where the climate is characterized by such temperature conditions. (See Huntington's *Civilization and Climate* (1).) In those lands where it remains above 64°F. all, or most all, of the time, or below 38°F., little development of humanity takes place unless the climate is possessed of great variability or storminess. Sufficiently wide and frequent changes in temperature, however, seem able to neutralize the depressive effects of heat on man. Unfortunately the large land and population masses in the tropics and sub-tropics are handicapped by both heat and lack of variability.

In certain of the most stimulating areas of the earth it seems evident that the climatic drive is exceeding man's bodily capacity to respond, with the result that in these regions metabolic breakdown is manifesting itself with increasing severity and frequency. The diabetes death rate, for instance, is steadily rising in spite of the free use of insulin, while the increase in diseases of the heart and blood vessels gives real cause for alarm.

It was in an effort to obtain a controlled experimental basis for the explanation of these climatic effects that we began our animal studies four years ago. We proposed to test on laboratory animals the effects of continuous moist heat, of continuous cold, and of a daily change from one to the

[1] This work, carried on for the last four years, was given generous financial support by the Frigidaire Corporation during the first two years, and later by the Union Central Life Insurance Company. Grants from the National Research Council and the Ella Sachs Plotz Foundation enabled us to carry on during the past year. To each of these donors we wish to express our appreciation and thanks.

other. The work is still incomplete, the present report being more or less preliminary. Certain positive results have been obtained, however, which warrant publication at this time.

Two rooms were constructed of steel and glass, properly insulated and lighted, and provided with continuous ventilation by an exhaust fan which drew air alike from both rooms. The rooms were eight feet square and seven feet high, and equipped with metal racks for animal cages. One room was kept at 88° to 92°F. by an electric radiant heater controlled by a bimetallic thermostat. Moisture in the air here was kept around 70 per cent saturation by an electrically heated shallow water pan fitted with an automatic filling device. An eleven inch fan blew air current across the water surface constantly, aiding evaporation and providing proper air movement within the room. The other room was cooled to 52° and 56°F. by a Frigidaire room cooler which contained a fan to provide air movement within the room similar to that in the hot room. No attention was paid to humidity in the cold room. Both rooms were equipped with recording clocks to give continuous charts of temperature, and in the hot room, of the relative humidity.

Rabbits, five to six months old and weighing three to five pounds, were placed in the rooms in three groups. One group was kept in the cold room for six weeks, a second for a similar period in the hot room, while the third group spent sixteen hours of each day in the hot room and eight hours in the cold room, for the six weeks' period. Purina Chow, oats and cracked corn were used as the principal food, with an addition of green vegetables twice a week. Only healthy animals were used in the final tests. Approximately equal numbers of male and non-pregnant female rabbits were used.

EXPERIMENTAL RESULTS. *Sensitivity to chilling.* After the three groups of animals had been subjected to their different environmental conditions for six weeks, representatives from each group were removed for testing. For each day's test we used rabbits as nearly alike as possible in weight, color, and nutritional state. For testing their ability to produce heat and maintain the body temperature under chilling conditions they were placed in a large water bath kept at 59°F. (15°C.) of such depth as to cover about half the body when they stood erect. Rectal temperatures were taken before placing them in the bath, and at frequent intervals during the chilling. Our interest centered mainly on the rate of fall of rectal temperature and the time required for them to reach such a degree of prostration as to be unable to keep the head above water. Several series of rabbits were tested in this way over a period of two years, with the following results.

	SERIES I	SERIES II	SERIES III	TOTAL NUMBER OF ANIMALS USED	MEAN TIME TAKEN TO REACH 26°C. RECTAL TEMPERATURE
Time (in minutes) required to reach rectal temperature of 77°F. (26°C.) Bath kept at 59°F. (15°C.)					
Hot room group.....	60, 75, 45, 45, 60 Average 57	60, 60, 60 Average 60	60, 60, 60, 50 Average 58	12	60.42±1.75
Cold room group.....	150, 90, 60, 60, 75 Average 87	150, 55, 90, 45, 210 Average 110	60, 120 Average 90	12	100.83±6.92
Group changed daily...	60, 135, 75, 75, 75, 60, 75 Average 79	40, 150, 120, 150, 90 Average 110	60, 85 Average 73	14	92.14±6.18
Survival time (in minutes) to complete exhaustion. Bath kept at 64.4°F. (18°C.)					
Hot room group.....	90	115	70	3	
Cold room group.....	160	135	90	3	
Group changed daily...	285	155	105	3	

In the tests carried out at 59°F. the differences between the mean for the hot room group and those for the other two groups are definitely significant, although this is not true of the difference between the cold room and changed group

$$\frac{\text{Difference } (100.83 - 60.42)}{\text{Probable Error of Difference}} = 5.66$$

$$\frac{\text{Difference } (92.14 - 60.42)}{\text{Probable Error of Difference}} = 4.94$$

$$\frac{\text{Difference } (100.83 - 92.14)}{\text{Probable Error of Difference}} = 0.94$$

At the time the rectal temperatures of the hot room animals reached 79°F. (26°C.) in the bath, the animals of the other groups were still in distinctly better condition. Actually little difference in behavior could be noticed here between the cold room and changed groups. When tested at 64.4°F. (18°C.) and left in bath to complete prostration, however, the group changed daily gave a longer survival time than did the cold room group. Although they had spent sixteen hours of each day in the hot room, they were more resistant to prostration by chilling than were those

that had lived all the time in the cold room. More will be said of this in a later paragraph.

Sensitivity to heat. In a way similar to the chilling tests described above, animals from the three groups were also tested for their ability to withstand high temperatures. Placing them in a warm water bath kept at 110°F. (43°C.) and noting the time required for prostration, the following data were obtained:

Animal from hot room group survived	110 minutes
Animal from cold room group survived	95 minutes
Animal changed between both rooms survived	80 minutes

The differences in resistance of the animals to heat were quite evident. The one adapted only to the moist heat of the hot room was able to delay longest the development of the terminal pyrexia and prostration. The one adapted only to the cold room was somewhat more susceptible to the heat, while the one shifted daily between the two rooms succumbed first of all.

Four animals from each of the three groups were tested in a different fashion for sensitivity to heat. They were placed in the hot room and the temperature level raised from the usual 88°–92°F. range to one of 96°–100°F. Here all those from the cold room group and those changed daily between the rooms were dead within 36 hours, while the survival times for the hot room group were 36, 88, 92 and 110 hours, respectively. Further discussion of these findings will come on a subsequent page.

Glycogen utilization. It was at first thought that the lessened ability of the hot room animals to keep warm might be dependent on insufficient stores of liver glycogen. However, we soon found that such was not the case. Although these rabbits ate less than the others, their liver glycogen remained high. Even after the chilling tests it did not show marked depletion. We therefore decided to compare quantitatively the rate of glycogen utilization under chilling conditions in the animals of the three groups. For this purpose a less severe method of chilling was employed.

In the first series tested, representatives were taken from the rooms and placed in out-of-door hutches without food for 24 hours. The mean temperature was 51°F. with a night minimum of 41°F. At the end of the 24 hours they were then killed by a blow on the head and the liver glycogen immediately determined (2). Controls from each group were removed from the rooms and killed without fasting, to get the normal liver glycogen content. The results, presented in table 1, show for the hot room animals a reduction in the average glycogen content from 5.99 grams, down to 1.85 grams during the chilling, a loss of 69 per cent. For the animals from the changed group there is seen almost complete exhaustion of liver glycogen. The reduction was from 6.69 grams down to 0.22 gram (average for the group), a loss of 96.7 per cent. Calculated in another way, the liver glyco-

gen percentage of the hot room group was reduced from 6.31 down to 4.17, a loss of 33.9 per cent, while for the changed group the reduction was from 6.57 down to 0.53, a loss of 91.9 per cent.

A second series of animals were tested by chilling at a somewhat higher temperature. They were placed in a room at 54°F. and kept there 24 hours

TABLE 1

Glycogen utilization under chilling conditions

SERIES I GROUP	SEX	LIVER						LOSS IN LIVER GLYCOGEN	
		Control animals not fasted or chilled			Animals fasted and chilled twenty-four hours			In weight	In percentage
		Weight	Glycogen		Weight	Glycogen			
		grams	*per cent*	*grams*	*grams*	*per cent*	*grams*	*per cent*	*per cent*
Hot room	M.	80.1	6.40	5.13					
	M.	109.0	6.12	6.68					
	M.	83.0	6.17	5.12					
	F.	116.6	6.70	7.81					
	F.	84.2	6.17	5.20					
Average		94.6	6.31	5.99					
Hot room	M.				35.5	5.19	1.84		
	M.				52.2	5.12	2.67		
	F.				47.3	5.12	2.42		
	F.				38.7	1.25	4.48		
Average					43.4	4.17	1.85	69.2	33.9
Changed group	F.	126.2	6.99	8.82					
	M.	83.1	6.22	5.17					
	F.	93.5	6.50	6.08					
Average		100.9	6.57	6.69					
Changed group	F.				42.5	0.19	0.08		
	F.				39.3	0.88	0.35		
	M.				42.5	0.52	0.22		
Average					41.4	0.53	0.22	96.7	91.9

without food and with moistened fur. Table 2 presents a summary of the data obtained from this series. With this less severe chilling, the glycogen utilization was less in each group, but the same relationships held as in the first series. The hot room animals used least, the changed group most, while the cold room group fell into an intermediate position. The differences in rate of glycogen loss in these two series are sufficiently marked as

to leave no room for doubt that there is a more rapid metabolism in the changed and cold room animals. The most significant feature of the results is the position assumed by the animals that endured the hot room environment 16 hours of each day but were cooled the remaining 8 hours. Here as in previous tests, this group showed evidence of a very active metabolism, even more active than that of the animals kept cooled at all times.

Adrenal glands. The weight of these glands was found to be lowest in the hot room groups, highest in the cold room animals, with the changed group taking an intermediate position. More complete statement of this phase of the study will be made later when more complete data are available.

TABLE 2

Glycogen utilization under chilling conditions

SERIES II	NUMBER OF ANIMALS	LIVER						LOSS IN LIVER GLYCOGEN	
		Controls animals not fasted or chilled			Animals fasted and chilled twenty-four hours			In weight	In percentage
		Weight	Glycogen		Weight	Glycogen			
		grams	*per cent*	*grams*	*grams*	*per cent*	*grams*	*per cent*	*per cent*
Hot room	5	50.9	2.96	1.60					
	2				54.7	2.04	1.14	28.7	31.1
Cold room	5	118.4	2.52	3.32					
	2				62.6	0.79	0.59	82.2	68.7
Changed room	5	71.5	4.42	3.28					
	3				54.8	0.82	0.46	86.0	81.4

Determinations of adrenalin (3) in the left adrenal of each animal killed showed no significant differences with the animals in the resting state. When submitted to the chilling in the cold bath, however, there was a definite tendency for the cold room and changed groups to show a greater fall in adrenalin content than was seen in the hot room animals.

Blood sugar. No significant differences were found in the blood sugar level between the various groups either at rest or during the chilling tests. Intramuscular injection of adrenalin produced slightly more of a hyperglycemia response in the hot room animals than in those of the other two groups but did not alter the survival time in the cold bath. All groups reacted about equally to insulin injections.

DISCUSSION. Marked adaptability of animal metabolism to changes in atmospheric environment are shown by the experimental data we have collected and present here in brief form. When animals are kept in a hot room with only slight temperature fluctuations, their metabolism becomes

sluggish and they lose in large measure the ability to meet chilling emergencies by an increased heat production. However by virtue of their low rate of combustion they are able to withstand temperatures much above the level to which they are adapted. Animals adapted to constant cold, on the other hand, have an active metabolism, stand chilling well, but stand high temperatures poorly.

Sundström, (4) working with rats, showed a definite difference in the level of heat production at different temperature levels. As the environmental temperature rose, heat production declined. This would not hold, of course, when over-heating takes place and the terminal pyrexia develops. Our results add significantly to those of Sundström in that we show the effects of the different environments on the animals' ability to meet sudden emergency changes. Our studies of liver glycogen show that it is not a question of available fuel which accounts for the varying response to chilling, but rather a difference in their ability to metabolize the glycogen present.

The most interesting part of our data concerns the effects of temperature *variability* on metabolism. A few hours of cooling each day is shown to completely overcome the depressing influence of a hot environment. Animals spending two-thirds of the time in the hot room but cooled a while each day, are found to have more active combustion than even the cold room ones. We believe these differences in metabolism under the various conditions to be mainly dependent on changes in adrenal function, secondarily influencing glycogen utilization. Definite and certain proof for this, however, is not yet at hand.

SUMMARY OF RESULTS

Animals adapted to constant heat are found to lose to a considerable degree their ability to produce heat and keep warm under chilling emergencies. Those adapted to a cooler environment are more able to meet chilling conditions by increased heat production, but succumb to excessive heat much more readily than do those adapted to constant warmth.

A few hours' cooling each day is found to counteract the depressive effects of heat, and to keep the metabolism just as responsive to chilling as does a constantly cool environment. Animals adapted to a cold or changeable environment rapidly exhaust their glycogen supplies when chilled, while combustion is much slower in those from the hot room.

BIBLIOGRAPHY

(1) HUNTINGTON, E. Civilization and climate. 3rd Ed. Yale University Press, New Haven, 1925.
(2) PFLÜGER, E. Pflüger's Arch., 1904, ciii, 169.
(3) FOLIN, O. AND H. TRIMBLE. Journ. Biol. Chem., 1924, lx, 472.
(4) SUNDSTROEM, E. S. Journ. Biol. Chem., 1925, lxiii (Proc.); 1927, lxvii, 320.

PHYSIOLOGICAL VARIATIONS IN THE GLUCOSE RATIO OF BLOOD AND CEREBROSPINAL FLUID

DAVID J. COHN, ABRAHAM LEVINSON AND FRANCES McCARTHY

From the Department of Chemistry, Nelson Morris Memorial Institute for Medical Research and the Sarah Morris Hospital for Children of the Michael Reese Hospital and the Children's Hospital of the Cook County Hospital

Received for publication October 22, 1932

The results of glucose determinations may vary in the same specimen of blood if different methods (such as the Lewis-Benedict, Folin-Wu and Shaffer-Hartmann) are used. This is due to the fact that the figures yielded by these methods represent not only the glucose present, but in addition, varying amounts of other substances, consisting mainly of glutathione, thionene, and compounds of the non-protein nitrogen group, which have the ability to reduce the oxidizing reagents used for the quantitative estimation of glucose (Thalhimer, 1922; Somogyi, 1926). The presence of these non-glucose reducing substances in blood has been demonstrated by several authors, and most recently by Somogyi, who has devised a modification of the Shaffer-Hartmann (1921) method by which the glucose alone, or, as it is often called, the "true" glucose, can be simply determined. In this method the use of an alkaline solution of a zinc salt causes the precipitation not only of the proteins but, at the same time, of practically all reducing substances, other than glucose. The filtrate, yielded by the zinc precipitation contains glucose as the only substance which affects the special copper solution subsequently used, and the amount of copper reduced is determined by iodiometric titration. Somogyi checked the results obtained by his method by determining the amount of material fermentable by yeast in the same specimens (Somogyi, 1927).

In our work we were concerned with the question as to whether or not similar non-glucose reducing substances occur in cerebrospinal fluid, and if so, to what extent. We were interested also in the relative levels of the glucose content of cerebrospinal fluid and of blood. This ratio has been much discussed in the literature, and we felt that it might be affected by the presence of the non-glucose reducing substances, which have not been considered until now in the interpretation of cerebrospinal fluid data.

METHOD AND MATERIAL. True glucose determinations were made by the method outlined by Somogyi. Non-glucose reducing substances were determined by subtracting these values from those obtained by the Folin-

Wu method on the same sample. We checked the results obtained this way by means of the yeast technique, which consists of glucose determinations before and after the action of washed yeast on the material to be analysed. The usual precautions concerning calibration of apparatus and frequent checking of solutions were rigidly followed.

The cerebrospinal fluid studied by us was obtained from hospital patients, mostly children, by spinal punctures, which were done only where indicated for diagnostic or therapeutic purposes. The data for patients suffering from meningitis, and a number of other diseases will be reported elsewhere. In this paper we are reporting only the normal values. Since we could not subject normal people to the necessary procedures, we selected as human "normals" those cases that proved to have no organic involvement of the central nervous system, and supplemented our studies with experiments on normal dogs.

In all human subjects the cerebrospinal fluid was obtained in the morning, from patients who had not ingested any food overnight, and to whom no drugs had been administered which might influence the glucose content of the body fluids.

In most instances, samples of blood were drawn simultaneously with the cerebrospinal fluid. The glucose determinations, on both blood and cerebrospinal fluid, were made immediately after the material was obtained, the specimens being kept cold during the interval before analysis. Sodium fluoride was added to the blood to prevent glycolysis.

Glucose in blood. In the course of our work on the glucose content of cerebrospinal fluid, we obtained data on the glucose in blood in a series of adults and children. In 100 non-diabetic adults, we found an average of 85 mgm. per 100 cc. with values ranging from 63 to 110 mgm. per 100 cc. This agrees with Somogyi's average of 87 mgm. per 100 cc. in adults. To our knowledge there is no report of the glucose content of the blood of children determined by the Somogyi method. In 54 children suffering from a variety of ailments, excluding meningitis and diabetes, we found an average blood glucose of 70 mgm. per 100 cc., with a range from 45 to 126 mgm. per 100 cc. Although these children were ill, they were generally in a good state of nutrition, and the rather low average glucose value comparable to that of adults could therefore not be accounted for on the ground of malnutrition.

Glucose in cerebrospinal fluid. The lowest glucose value we obtained in our series of non-meningitic cases was 38 mgm. per 100 cc. and the highest was 75 mgm., with an average of 55 mgm. per 100 cc. of cerebrospinal fluid.

Non-glucose reducing substances in cerebrospinal fluid. We found that non-glucose reducing substances are present in the cerebrospinal fluid of normal children, but usually to a lesser extent than in blood. Calculated as glucose, they were present in amounts up to 17 mgm. per 100 cc. of

cerebrospinal fluid, with an average of 8 mgm. This is approximately 40 per cent of the corresponding average figure for blood. Somogyi (1926). In two cases, the non-glucose reducing substances in the cerebrospinal fluid was somewhat higher than in the blood.

The same proportions have been found to be true in a small series of fluids obtained from adults.

Comparative glucose content of blood and cerebrospinal fluid. The relation between the glucose content of blood and cerebrospinal fluid has been studied by a number of authors, with the general conclusion that the cerebrospinal fluid sugar is between 45 and 80 per cent of the blood glucose.

Thus Seham and Nixon (1921) state that their results show "That the

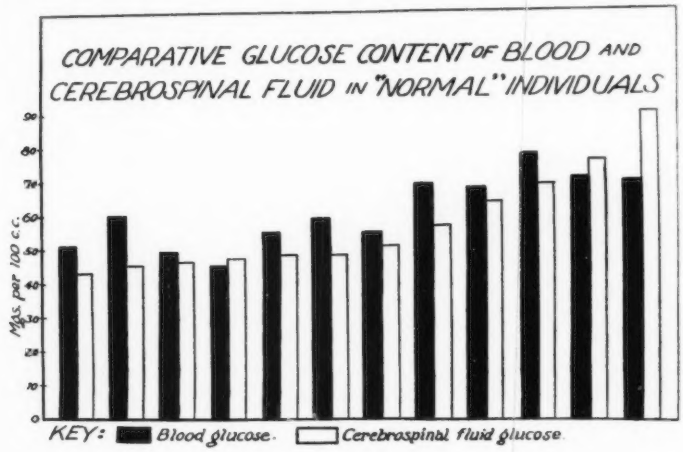

COMPARATIVE GLUCOSE CONTENT OF BLOOD AND CEREBROSPINAL FLUID IN "NORMAL" INDIVIDUALS
MGS. PER 100 C.C.
KEY: Blood glucose. Cerebrospinal fluid glucose.

Fig. 1

ratio between the spinal fluid and blood of normal individuals is 56.2 with a maximum of 70.3 and a minimum of 47.8."

According to Goodwin and Shelley (1925), "There is a definite relationship between the cerebrospinal fluid sugar and the blood sugar, and the figure representing this relationship, stated as the percentual relationship between the two, is relatively constant, lying between 45 and 65 per cent, provided determinations are made on both blood and cerebrospinal fluid taken at the same time, after a night's fasting."

Wilcox and Lyttle (1923) find that, "Normally the spinal fluid sugar concentration is about one-half that of the blood, and its range of variation corresponds to that of the blood."

In a previous publication, using the Folin-Wu method, Levinson (1925, 1929) found the cerebrospinal fluid to contain from 50 to 75 per cent as much glucose as the blood. The general consensus of opinion expressed

in the literature is that the cerebrospinal fluid sugar under normal conditions never exceeds that of the blood.

In the present study we have found the glucose content of cerebrospinal fluid to be much nearer that of the blood than was previously believed to be true and furthermore that it is possible for the cerebrospinal fluid glucose content to be the same or even higher than of blood (fig. 1).

TABLE 1

Ratios of cerebrospinal fluid glucose to blood glucose calculated on the basis of results obtained by the Folin Wu and Somogyi methods

CASE NUMBER	FOLIN WU			SOMOGYI		
	Cerebrospinal fluid glucose	Blood glucose	Ratio	Cerebrospinal fluid glucose	Blood glucose	Ratio
			per cent			*per cent*
1	54	78	68	51	55	93
2	76	128	59	62	88	71
3	76	100	76	64	68	95
4	100	118	85	90	70	128
5	77	104	74	73	73	100
6	58	74	78	43	51	84
7	62	80	78	45	56	81
8	66	104	64	61	80	76
9	56	79	71	45	60	75
10	56	90	62	48	67	72
Average.....			71.5			87.6

Our explanation for this difference is that when the older methods of glucose determination are used the ratio determined is, in fact, not

$$\frac{\text{glucose in cerebrospinal fluid}}{\text{glucose in blood}}$$

but

$$\frac{\text{glucose in cerebrospinal fluid} + (\text{about 8 mgm. of non-glucose reducing substance})}{\text{glucose in blood} + (\text{about 25 mgm. of non-glucose reducing substance})}$$

The part of the ratio enclosed in the parentheses necessarily diminishes the quotient and is responsible for the low ratios reported in the literature, and for the impression that the cerebrospinal fluid glucose cannot under normal fasting conditions be as great or greater than that of the blood.

Our results, therefore, may be explained by the fact that we have eliminated the factor of the non-glucose reducing substances which are present in greater amounts in blood than in cerebrospinal fluid. This is illustrated by table 1, in which the ratios are calculated from results obtained by both the Somogyi and the Folin-Wu methods (table 1).

Animal experiments. Our determinations on the cerebrospinal fluid and blood of human subjects indicate, as pointed out above, that in the fasting state the glucose content of the cerebrospinal fluid may be less, the same, or greater than in blood. In order to verify this point, and, if possible, to explain how these conditions are brought about, we undertook a series of experiments upon normal dogs.

The animals were anesthetized with nembutal,[1] (a substance which we have found to have a negligible effect on the glucose content of the blood), and the glucose contents of both the cerebrospinal fluid and venous blood were determined at half-hourly intervals for several hours.

To one group of dogs glucose was administered, either by stomach tube or intravenously. Figure 2 is a representation of a typical experiment (fig. 2). It will be seen from this figure that the rise in glucose in the blood

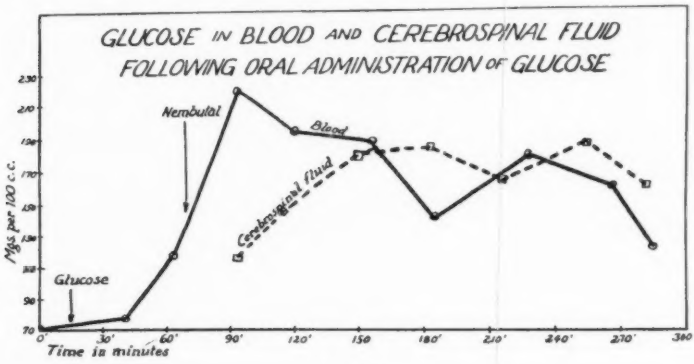


Fig. 2

subsequent to the administration of the sugar is followed by a similar rise in the cerebrospinal fluid, but that the rate of increase of the glucose level in the cerebrospinal fluid is much slower. Furthermore, when the glucose content of the blood has reached its peak value, and begins to fall, the cerebrospinal fluid glucose does not change correspondingly, but lags behind. As a result, a point is reached at which the glucose content of the blood has fallen below that of the cerebrospinal fluid. Thus it is easily seen how, sometime after glucose administration, there is a period during which the cerebrospinal fluid may contain a considerable higher percentage of glucose than that of the blood.

However, even without glucose administration, there is a sufficient normal physiological variation in the glucose content of the blood to bring about relationships, analogous to the above, between the glucose content

[1] Kindly supplied by the Abbott Laboratories.

of the blood and that of the cerebrospinal fluid. Wavelike changes in the blood glucose of fasting subjects were noted by Hansen (1923) and confirmed by us in a series of determinations on dogs (some under nembutal anesthesia, and some unanesthetized). We have found half-hourly variations of from 15 to 20 mgm. per 100 cc., which is well outside of our experimental error in sugar determinations. Such wavelike variations in the glucose content of the blood in fasting dogs, without administration of glucose, are depicted in the curves in figure 3, together with the corresponding curves for the sugar in cerebrospinal fluid drawn simultaneously. It will be seen that here, too, the cerebrospinal fluid glucose is at times the same, or even higher than the blood glucose. In fact, the curves are similar in general character to those after glucose administration, except that the fluctuations are not so great. The glucose content of the cerebrospinal

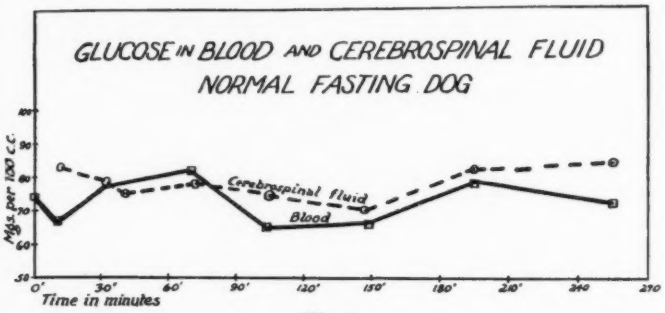


Fig. 3

fluid has a tendency to follow the variations taking place in the blood of fasting animals, but due to the lag in reaching equilibrium, the same conditions are obtained as after glucose administration, i.e., the cerebrospinal glucose curve is at times higher than the blood glucose curve.

The above, we believe, explains our finding that individual specimens of cerebrospinal fluid obtained from normal fasting patients may be less, the same, or even greater than that of blood which is drawn simultaneously.

SUMMARY

1. The true glucose content of cerebrospinal fluid in normal fasting human subjects usually approximates that of blood. It may, however, be less or more, depending upon the fluctuations in the blood sugar.

2. Glucose determinations on samples of blood and cerebrospinal fluid drawn simultaneously at short intervals over a period of several hours from fasting dogs and dogs to whom glucose had been administered checked the results obtained in human subjects.

BIBLIOGRAPHY

FOLIN, O. AND H. WU. 1919. Journ. Biol. Chem., xxxviii, 106.
GOODWIN, G. M. AND H. J. SHELLEY. 1925. Arch. Int. Med., xxv, 242.
HANSEN, K. M. Acta Med. Scand., 1923, i, Suppl. iv.
LEVINSON, A. 1925. Amer. Journ. Dis. Child., xxx, 774.
1929. Cerebrospinal fluid in health and in disease. 3rd. ed., C. V. Mosby Co., St. Louis.
LEWIS, R. C. AND S. R. BENEDICT. 1915. Journ. Biol. Chem., xiv, 349.
SOMOGYI, M. 1926. Journ. Biol. Chem., lxx, 599.
1927. Journ. Biol. Chem., lxxv, 33.
THALHIMER, W. AND UPDEGRAFF. 1922. Journ. Amer. Med. Assoc., lxxviii, 1383.
WILCOX, H. B. AND J. C. LYTTLE. 1923. Arch. Pediat., xi, 215.

THE EFFECTS OF SUGAR AND ELECTROLYTE SOLUTIONS ON THE METABOLISM AND IRRITABILITY OF HEART MUSCLE

JOSEPH VICTOR

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City, and the Department of Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Received for publication November 8, 1932

Considerable evidence has recently accumulated relating irritability to the metabolic rate of the skeletal muscle of the frog. In skeletal muscle the metabolic response varies with the state of irritability of the muscle. It therefore seemed of interest to investigate the influence of various substances on the oxygen consumption and irritability of the frog's cardiac muscle. The reagents studied were sucrose, glucose, Ringer's solution, KCl, $CaCl_2$, NaCl, HCl, and methylene blue.

METHOD. The oxygen consumption measurements were done by means of the Fenn modification of the Thunberg differential volumeter (12). Each bottle had a capacity of about 15 cc. The capillary volume was about 2.5 cmm. per centimeter. The experimental bottle was fitted with two side-arms set at right angles to one another. The smaller one held a few drops of NaOH solution to absorb CO_2 and the larger contained the substance whose effect was to be determined. In all experiments the tissue was placed in the bottle without any solution bathing it. Four-tenths of a cubic centimeter of the reagent to be investigated was placed in the side-arm and tipped on and off the tissue at will. The bottles were filled with pure oxygen but were not shaken.

The cardiac ventricular muscle of Rana pipiens and Rana clamitans was used. The ventricle of the heart was removed along the atrio-ventricular groove and cut longitudinally through the apex into two parts of as nearly equal size as possible. The tissue was blotted gently with filter paper to remove the adherent blood. After weighing, the muscle was placed in the experimental bottle either immediately or after soaking in Ringer's solution for about one-half hour. The temperature was approximately 22.5 degrees Centigrade in every instance and was controlled to within 0.01 degree in any one experiment. The tissues weighed between 20 and 40 mgm. The Ringer's solution contained NaCl 0.65 per cent, KCl 0.0075 per cent, and $CaCl_2$ 0.01 per cent. No buffer was used. The tested reagents were made up in isotonic solutions, unless otherwise noted.

The irritability of the muscle was determined by a chronaximeter using the condenser discharge method of Lapique. The resistances were arranged with 17,000 ohms in series with the tissue. The latter was shunted by a 3,000 ohms resistor so that variation of the tissue resistance between 2,000 and 50,000 ohms varied the total resistance by not more than 15 per cent from 20,000 ohms. The electrodes were Ag–AgCl, freshly coated every day and constantly 5 mm. apart. A microammeter was placed in series with the condensers and the electrodes so that the current discharged by the condensers through the electrodes could always be observed. Most of the irritability experiments were performed separately from those on the oxygen consumption and were done in air at room temperature; however, a saturated vapor pressure was maintained by the presence of 0.4 cc. of the test solution in the chamber. The tissue was placed on the electrodes at various time intervals. Exactly the same position of the muscle on the electrodes could not be duplicated. Replacing the tissue on the electrodes results in slight variation in rheobase and chronaxie. But this variation is about one-tenth that observed following the addition of the test substances to the tissue. These experimental procedures were as nearly as possible duplicates of those on the oxygen consumption.

The experiments were begun in January, 1930, and continued through the year without noting any obvious seasonal variation.

Sugar solutions. It was first shown by Overton (21) that the non-irritability produced by soaking skeletal muscle in isotonic sugar solution occurred within 15 minutes and was reversed by isotonic NaCl or by 0.1 per cent NaCl in 6 per cent sucrose. This non-irritability was later found by Embden and Lange (9) to be associated with an increased phosphoric acid excretion and oxygen consumption, which were decreased by Ringer's solution. Fenn (13) has found this non-irritability to be also associated with a slight contraction of the muscle, an increased lactic acid concentration, and an electrical change in the muscle that is temporarily negative but predominantly positive. The increased phosphoric acid excretion has been shown by Cardosa (3) to be secondary to the splitting of phosphagen that is accelerated by sucrose. Furthermore, the spontaneous non-irritability described by Duliére and Horton (8) was shown by Horton (17) to be accompanied by an increased potassium excretion. Fenn (11) found this to be also accompanied by an increased rate of oxygen consumption that was decreased by Ringer's solution.

On the other hand, skeletal muscles spontaneously non-irritable or those that have lost their irritability by soaking in isotonic K_2SO_4 do not show any change when placed in sugar solutions (13). It seems, therefore, that the changes occurring during the loss of irritability due to sugar solution are associated with an increased metabolic rate, whereas sucrose solutions have no effects on the total metabolism of skeletal muscle already non-irritable.

The addition of isotonic sucrose to cardiac ventricular muscle results in a fall in the rate of oxygen consumption. This is shown in figure 1.

The point where the sugar solution is tipped on the tissue previously bathed in Ringer's solution for 40 minutes following its dissection, is marked by the arrow. When sucrose is added to unwashed non-irritable heart muscle there is a similar fall in metabolic rate. This is also seen in figure 1. In the latter experiment the muscle had been removed 5 hours before the addition of the sugar solution.

In a series of experiments on the irritability of unwashed cardiac muscle, spontaneous non-irritability occurred in 20 to 100 minutes. The onset of spontaneous non-irritability is indicated by a rise in chronaxie and rheobase. Non-irritability, that is, the absence of any response to a stimulus of 80 volts discharged through 3 microfarads and 20,000 ohms, or 80 volts

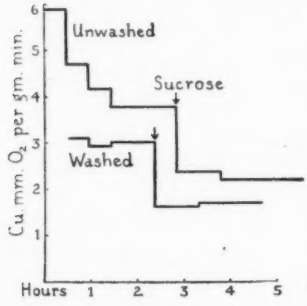


Fig. 1

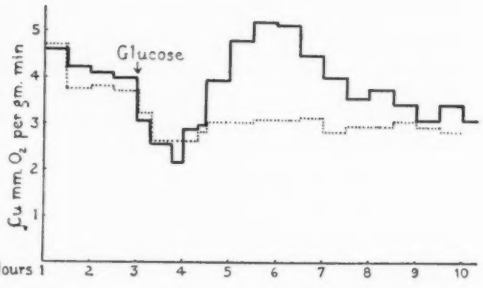


Fig. 2

Fig. 1. The depression of the rate of oxygen consumption of unwashed and Ringer-washed cardiac ventricular muscle by isotonic sucrose solutions.

Fig. 2. The effects of isotonic glucose solution on the metabolic rate of unwashed cardiac ventricular muscle. The dotted curve represents the exceptional reaction referred to in the text.

for the duration of 22.5 sigma, resulting from the addition of isotonic sucrose to irritable cardiac muscle, is also reached in approximately the same time and is approached by a similar rise in chronaxie and rheobase. The muscle tone seems to decrease, for the tissue appears soft and flaccid after about two hours in sucrose solution. However, the metabolic changes due to sucrose solution take place immediately. *In contradistinction to voluntary muscle the metabolic reaction of cardiac muscle to sucrose is uninfluenced by its state of irritability. Moreover, its change in metabolic rate is opposite that of irritable skeletal muscle.*

As already stated, the isolated ventricular muscle loses its irritability within 2 hours if it is kept in a chamber saturated with water vapor. In none of the 81 experiments, where the tissue has not been soaked in

Ringer's solution, has there been any spontaneous increase in the rate of oxygen consumption within this period of time. Before testing any reagent the metabolism, in every instance, was followed for at least 2 and in some experiments for 8 hours.

The depressive influence of isotonic sucrose solution on the metabolic rate is not due to a change in oxygen tension about the tissue when the fluid is added. Tipping the respirometer so that the bathing solution flows back again into the side-arm does not alter the effect of the sucrose.

The effects of isotonic glucose are different from those of sucrose. When unwashed ventricular muscle is placed in glucose a fall in the oxygen consumption is observed, lasting about 2 or 3 hours. This is followed by a rise of about the same duration and then the metabolism returns to the normal level. (See fig. 2.) These results were obtained in 5 of 6 experiments. The exception in this group showed a reduction in metabolic

TABLE 1

Cardiac muscle

Kept in NaCl	Spontaneous non-irritability		Kept in Ringer's
↓	↓	↙	↓
Non-irritable in 45 minutes	NaCl for 5 minutes	Irritable indefinitely	Kept in NaCl
	↓		↓
	Irritable 15 minutes		Irritable 6 hours
	↓		↓
	Non-irritable		Non-irritable
	↓		
	Kept in Ringer's		
	↓		
	Irritable indefinitely		

rate similar to that obtained with sucrose. Non-irritable muscle placed in glucose solution shows no further change in irritability. The non-irritability produced in glucose in about 2 hours is also found to be reversed in Ringer's solution.

Sodium chloride. Isotonic NaCl alone has no influence on the oxygen consumption of irritable skeletal muscle (11) but lowers the metabolic rate in non-irritable sugar soaked muscle. The spontaneous non-irritability of voluntary muscle is permanently reversed by 0.65 per cent NaCl solutions (8). Spontaneously non-irritable ventricular muscle occasionally shows a temporary recovery, after about 15 minutes in NaCl solution, lasting approximately 15 minutes. Heart muscle in NaCl solution loses its irritability in about 45 minutes after dissection. However, muscle previously soaked in Ringer's solution for about an hour maintains its irritability in isotonic NaCl for about 6 hours. Ringer (22) described prolongation of diastole and systole with a loss of contractility of the heart

after 20 minutes in 0.75 per cent NaCl. Spontaneous contractions disappeared in about 20 minutes in our experiments, yet the muscle responded to electrical stimulation, but with a rather high chronaxie and rheobase, for about 25 minutes longer. Following NaCl treatment, non-irritability is reversed by Ringer's.

Table 1 shows the treatment and change in irritability.

The rate of oxygen consumption in Ringer-washed irritable cardiac muscle is slightly depressed or is not at all affected by isotonic NaCl (fig. 3). In spontaneously non-irritable muscle the oxygen consumption is usually slightly depressed, though in 2 cases out of 6 the metabolism was slightly increased.

Potassium chloride. In 1924 Griffith (15) showed that the acid and CO_2 production of frog skeletal muscle was temporarily greatly increased by

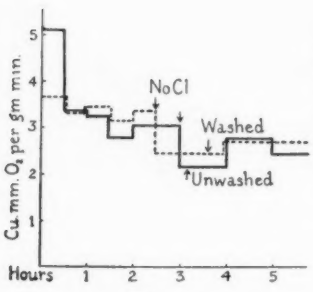


Fig. 3

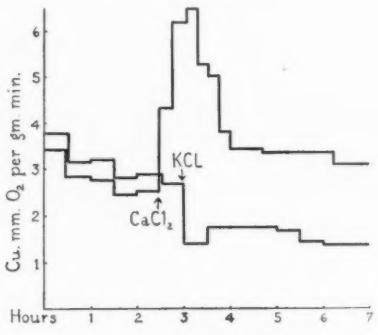


Fig. 4

Fig. 3. The effect of isotonic NaCl on the metabolic rate of unwashed and Ringer-washed cardiac ventricular muscle. The dotted curve is that of the Ringer-washed tissue.

Fig. 4. The antagonistic action of isotonic solutions of KCl and $CaCl_2$ upon the rate of oxygen consumption of cardiac ventricular muscle.

isotonic KCl solutions, whereas that of frog heart and turtle ventricle was immediately and permanently decreased. Barcroft and Dixon (1), studying the influence of various substances on the metabolism of beating hearts of dogs and cats, found that small amounts of KCl decreased the oxygen consumption more than the CO_2 production. They thought that potassium probably caused some alteration of the tissue in the nature of decomposition.

The increased rate of oxygen consumption of irritable skeletal muscle due to potassium salts, has been shown by Fenn (12) to be accompanied by a contracture. In spontaneously non-irritable muscle and muscle made non-irritable with sugar, the rise in oxygen consumption and the con-

tracture due to potassium are prevented. Instead of a rise there is a slight fall in metabolic rate.

Isotonic KCl causes a fall in the rate of oxygen consumption in both Ringer-washed irritable and unwashed non-irritable heart muscle (fig. 4). The change of oxygen consumption parallels that seen in Griffith's curves for the CO_2 production of cardiac muscle.

KCl affects the rate of oxygen consumption of cardiac muscle, nerve (14), mammalian kidney, (20), and spontaneously or sugar treated non-irritable skeletal muscle (13) in the same way. *However, the rate of metabolism of irritable skeletal muscle is increased by KCl and is thus opposite to that of heart ventricle.* Fenn has found a parallelism between the rate of oxygen consumption and the phosphoric acid excretion under the influence of potassium and sugar solutions. But the electrical responses of skeletal muscles under the influence of sugar seem to be opposite to those obtained with potassium.

The spontaneous non-irritability of cardiac muscle is not altered by KCl. The addition of isotonic KCl to Ringer-soaked heart muscle results in a loss of irritability within 3 minutes. This is reversed in about 10 minutes by placing the non-irritable muscle in Ringer's when both chronaxie and rheobase increase. The muscle becomes soft and flaccid in the KCl solution. Ringer (22, 23), Sakai (24), Kisch (18), and others found that potassium salts paralyze and decrease the contractility of frogs' hearts. This change the former observed to be reversed by blood.

The action of KCl on the contractility and frequency of the beat of frog's hearts has been noted by investigators (22, 23, 24, 18) to be similar to but stronger than that of NaCl. The same trend is found to obtain regarding oxygen consumption and irritability.

Calcium chloride. Fenn (13) observed that the oxygen consumption of irritable or sugar-soaked skeletal muscle was decreased by isotonic $CaCl_2$. The bivalent cations Ca, Sr, Ba, and Mg in small amounts have been shown to cause an increase in the metabolic rate of mammalian kidney tissue (20). Gerard (14), working with frog nerve and Hess (16) with Planaria, observed that $CaCl_2$ decreased the oxygen consumption of these tissues.

From the accompanying graph (fig. 4) it is seen that *treating spontaneously non-irritable or Ringer-washed irritable cardiac muscle with $CaCl_2$ solution causes a sharp rise of the metabolic rate to between 50 and 200 per cent above normal, an effect opposite to that observed in skeletal muscle.* This increased rate is not permanent, however, lasting about 5 hours in unwashed non-irritable heart muscle and about 2 hours in Ringer-soaked muscle. Examination after removal from the $CaCl_2$ solution reveals a contracted, firm muscle with its cut edges rolled toward one another. It is not irritable.

When Ringer-washed irritable cardiac muscle is placed in $CaCl_2$ solution

there is a rise in its chronaxie and rheobase. Within 30 minutes it becomes non-irritable. Its latent period is prolonged and relaxation after contraction proceeds very slowly. Increased contractility and prolonged dilatation of cardiac muscle in Ca solutions were first reported by Ringer (23). Heart muscle placed in $CaCl_2$ solution immediately after dissection reacts the same as it does when placed in $CaCl_2$ after being soaked in Ringer's. The non-irritability produced by immersion in calcium solutions for about 15 minutes is reversed in Ringer's in approximately 5 hours. The return of irritability is gradual, as evidenced by a diminishing chronaxie and rheobase.

Spontaneously non-irritable cardiac muscle placed in isotonic $CaCl_2$ shows a temporary recovery within 3 minutes and lasting about 7 minutes. Both the rheobase and chronaxie are about twice normal during this period of excitability.

Ringer's solution. As already mentioned, the addition of Ringer's solution to sugar soaked or spontaneously non-irritable skeletal muscle lowers the increased metabolic rate to normal. Ringer's has no influence, however, on the rate of oxygen consumption of irritable voluntary muscle (13). The addition of Ringer's to spontaneously non-irritable heart muscle lowers the respiration slightly. Cardiac muscle washed in Ringer's solution reaches a steady rate of metabolism much sooner than does unwashed muscle. Ringer's added to irritable cardiac muscle previously washed in Ringer's has no influence on the oxygen consumption.

The addition of Ringer's solution to cardiac muscle rendered non-irritable by soaking in isotonic solutions of NaCl, KCl, sucrose and glucose causes a reversal of irritability within 15 minutes with a decrease in chronaxie and rheobase in all instances. The non-irritability and contracture produced by $CaCl_2$ requires several hours for reversal.

In several experiments either the KCl or $CaCl_2$ was omitted from the Ringer's solution. Calcium free Ringer's slightly diminished the oxygen consumption of spontaneously non-irritable cardiac muscle in 2 of 4 experiments, and had no metabolic effect in the other 2. The metabolic rate of Ringer-soaked cardiac muscle is uninfluenced by immersion into potassium or calcium free Ringer's solution. Potassium free Ringer's solution slightly diminished the oxygen use of spontaneously non-irritable heart muscle in 2 experiments. However, *spontaneous non-irritability is reversed by potassium free but not by calcium free Ringer's solution.* This reaction is different from that observed in skeletal muscle (8) in which irritability is permanently restored by calcium free Ringer's solution. *The irritability of Ringer-washed cardiac muscle is lost in calcium free, but only slightly diminished in potassium free Ringer's.* The loss of irritability of cardiac muscle in calcium free Ringer's is not due to changes in the phosphagen content of the muscle (7). Clark and White (6) found that Ringer's solution con-

taining low calcium or high potassium concentrations, or lacking potassium diminished the oxygen consumption of beating hearts in proportion to the reduction of work done by the heart. Since the heart muscle did no work under the conditions of these experiments, the changes observed are not due to alteration of mechanical response. The influence of these ions on the irritability of the heart has been widely discussed (22, 21, 18).

Perhaps tabulation of the effects of the ions discussed above on cardiac muscle and voluntary muscle may clarify the above results. This has been done in table 2.

TABLE 2

Effects of various solutions on the metabolic rate and irritability of cardiac and voluntary muscle

ISOTONIC SOLUTIONS	CONDITION OF MUSCLE BEFORE TESTING EFFECT OF SOLUTION							
	Irritable				Non-irritable			
	Oxygen consumption		Irritability		Oxygen consumption		Irritability	
	Ventricular muscle	Skeletal muscle	Ventricular muscle	Skeletal muscle	Ventricular muscle	Skeletal muscle	Ventricular muscle	Skeletal muscle
Sucrose	=	++	0	−	=	−	0	0
NaCl	−	0	−	0	−	=	±	+
KCl	=	++	−	C.	=	=	0	
$CaCl_2$	++	=	C.		++	−	± C.	
Ringer	0	0	0	0	−	=	+	+
Ringer-K	0		0	0	−		+	
Ringer-Ca	0		−		−		0	+

(−) = decreased; (+) = increased; (0) = no effect; (=) = markedly decreased; (++) = markedly increased; (0) = slightly decreased; (±) = temporarily increased, then return to non-irritability; C. = contracture.

Several facts become apparent from the table. The effects of isotonic solutions of sucrose, KCl and $CaCl_2$ on the metabolic rate of heart muscle are the opposite to those obtained with skeletal muscle. Furthermore, the effects of these substances cannot be correlated with the previous state of irritability of cardiac muscle, but do vary with the state of irritability of voluntary muscle; for example, KCl solution increased the oxygen consumption of irritable skeletal muscle, but decreased that of non-irritable skeletal muscle and of irritable or non-irritable cardiac muscle.

Potassium and calcium are antagonistic in the same muscle, but in the types of muscle under discussion their antagonism has an opposite direc-

tion, i.e., potassium decreases the tone and metabolic rate of heart muscle and increases these properties of skeletal muscle.

Although these solutions produce non-irritability in irritable cardiac muscle the loss of irritability occurs in different ways. Sucrose does not hasten the loss of irritability of heart muscle as compared with the onset of spontaneous non-irritability, whereas KCl produces non-irritability in very few minutes. However, the appearance of the muscle during the state of non-irritability is the same in either case—it becomes soft, relaxed, and flaccid. The duration of the time of onset of non-irritability produced by $CaCl_2$ solutions is greater than that necessary for producing KCl non-irritability and less than the time required for producing non-irritability by sucrose. Furthermore, $CaCl_2$ solution causes a firm and contracted heart muscle. Sucrose and KCl solutions do not affect the gross appear-

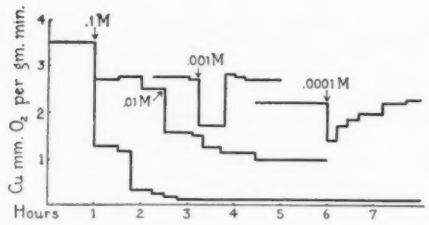


Fig. 5

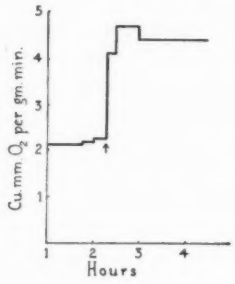


Fig. 6

Fig. 5. The effects of various concentrations of HCl upon the metabolic rate of cardiac ventricular muscle.

Fig. 6. The increased metabolic rate due to 0.01 per cent methylene blue in Ringer's solution. The addition of this solution is indicated by the arrow.

ance or reverse the non-irritability of spontaneously non-irritable cardiac muscle, whereas $CaCl_2$ causes a temporary recovery of irritability lasting about 5 to 7 minutes, at the end of which period the ventricular muscle is firm and contracted. Other differences between skeletal and cardiac muscle already discussed are found in the reactions to potassium free and calcium free Ringer's solution.

Acid. There has been considerable investigation of the influence of acid on the oxygen consumption of tissues. Below pH 7 the respiratory rate of liver and minced heart or skeletal muscle of the rabbit is decreased (19). Fenn reports that HCl in a concentration of 0.002 M has no effect on the metabolic rate, whereas stronger concentrations (0.01 to 0.2 M) increase it. This increased metabolism is associated with a contracture. In the contraction produced by 0.2 M there is a temporary rise in oxygen consumption followed by a fall probably due to the injurious effects of the acid on the oxidizing system (12).

In figure 5 are shown graphs of experiments in which HCl of various concentrations in NaCl was tipped on unwashed cardiac muscle. Concentrations of 0.0001 and 0.001 M have no influence on the respiratory rate. The 0.01 M solution depresses the rate to less than one-half normal, whereas 0.1 M solution almost completely inhibits respiration. Brody (2) has reported the action of 0.4 M HCl on heart ventricle. This concentration has the same effect as a 0.1 M solution on the metabolic rate.

The metabolic response of cardiac muscle to various concentrations of acid is different from that of voluntary muscle. In no case was an increase observed in the respiration of the heart. Furthermore, the concentration that depresses the respiration of cardiac muscle to less than one-half, increases the respiration of skeletal muscle. In the concentrations used no contracture is observed in cardiac muscle.

Although the acid was in isotonic NaCl solutions when added to the muscles, the tissues treated with 0.001 M or 0.0001 M HCl were irritable at the end of the experiment whereas those in stronger concentrations or in saline were non-irritable. The presence of dilute HCl (0.001 M and 0.0001 M) in isotonic saline reversed spontaneous non-irritability and maintained irritability for at least three hours. Observations were not made beyond this point.

Methylene blue. The rate of oxygen consumption of nerve (14), skeletal muscle (12) and other tissues is greatly increased by methylene blue. The addition of 0.01 per cent methylene blue in Ringer's solution to heart muscle increases the oxygen consumption 100 to 200 per cent (fig. 6).

SUMMARY AND CONCLUSIONS

Isotonic solutions of KCl and sucrose depress the oxygen consumption of irritable or non-irritable cardiac muscle. This is associated with a decrease in muscle tone and loss of irritability. Isotonic $CaCl_2$ increases both the tone and metabolic rate of irritable and non-irritable heart muscle, but renders the irritable muscle non-irritable. The antagonistic action of KCl and sucrose and of $CaCl_2$ on the metabolic rate and tone of heart muscle is found to be opposite that of irritable skeletal muscle. Furthermore, the action of these substances is independent of the previous state of irritability of heart muscle but varies with the previous state of irritabi ity of skeletal muscle. If spontaneous sugar or potassium non-irritability in cardiac muscle is due to the same causes as obtain in skeletal muscle the similarity of the action of sucrose and potassium solutions may be due to the washing away of calcium by the sucrose solution and thus increase the K/Ca ratio at the surface membranes of the muscle fibers. Furthermore, if the differences observed are due to membrane equilibria it might be suggested that heart muscle differed from skeletal muscle in having its membranes reversed and that a similar reversal of equilibrium

was responsible for the phenomenon of non-irritability in skeletal muscle. Much more study, however, is required before theorizing in these matters will be profitable.

The metabolic rate of non-irritable cardiac muscle first decreases, then after several hours increases, and finally returns to normal after immersion in an isotonic solution of glucose.

Ringer's solution slightly depresses the oxygen consumption of spontaneously non-irritable cardiac muscle, but has no effect on the metabolic rate of irritable heart muscle.

HCl in concentrations above 0.01 M in isotonic NaCl decreases the oxygen consumption of spontaneously non-irritable heart muscle.

Methylene blue increases the oxygen consumption of irritable and non-irritable heart muscle.

I am indebted to Prof. Wallace O. Fenn for his kind interest and helpful suggestions throughout the course of these experiments.

BIBLIOGRAPHY

(1) Barcroft and Dixon: Journ. Physiol., 1906, xxxv, 182.
(2) Brody: This Journal, 1930, xciii, 190.
(3) Cardoso: Pflüger's Arch., 1931, ccxxviii, 409.
(4) Clark: Proc. Roy. Soc. Med., 1912, v.; Therap. and Pharm. Sec., 181.
(5) Clark: Journ. Physiol., 1928, lxvi, 85.
(6) Clark and White: Journ. Physiol., 1928, lxvi, 203.
(7) Clark, Eggleston and Eggleston: Journ. Physiol., 1931, lxxii, 25P.
(8) Dulière and Horton: Journ. Physiol., 1929, lxvii, 153.
(9) Embden and Lange: Zeitschr. f. Physiol. Chem., 1923, cxxv, 258.
(10) Fenn: This Journal, 1927, lxxx, 327.
(11) Fenn: This Journal, 1930, xciii, 124.
(12) Fenn: Journ. Pharm. Exper. Therap., 1931, xlii, 81.
(13) Fenn: This Journal, 1931, xcvii, 635.
(14) Gerard: Proc. Soc. Exp. Biol. and Med., 1930, xxvii, 1052.
(15) Griffith: Journ. Gen. Physiol., 1924, vi, 683.
(16) Hess: Physiol. Zool., 1930, iii, 8.
(17) Horton: Journ. Physiol., 1930, lxx, 389.
(18) Kisch: Hand. d. Norm. u. Pathol. Physiol., 1926, vi, 712.
(19) Koehler and Reitzel: Journ. Biol. Chem., 1925, lxiv, 739.
(20) Leibowitz: Biochem. Zeitschr., 1930, ccxxvi, 338.
(21) Overton: Pflüger's Arch., 1902, xcii, 346.
(22) Ringer: Journ. Physiol., 1882, iii, 195, 380.
(23) Ringer: Journ. Physiol., 1883, iv, 29.
(24) Sakai: Zeitschr. f. Biol., 1914, lxiv, 505.

THE CONTINUED INJECTION OF OESTRIN INTO YOUNG RATS

GREGORY PINCUS AND N. WERTHESSEN

From the Laboratory of General Physiology, Harvard University, Cambridge, Massachusetts

Received for publication November 4, 1932

Young rats of a long-inbred, homogeneous strain (strain *A*, see: Crozier and Pincus, 1931) were injected with various amounts of oestrin (Theelin)[1] for various lengths of time onward from the 21st day after birth. Weights of ovaries, anterior pituitaries, posterior pituitaries, and thyroids were taken at various intervals after injection, and in uninjected controls. The data are summarized in table 1. Each group of controls and injected animals consisted of five animals (in two instances only four animals were used). Injections (subcutaneous) were carried on for 5, 10, 20, 30, and 40 days as indicated. The animals were killed 24 hours after the last injection. The glands were dissected out under a neutral oil, and carefully weighed on a Kuhlman microbalance insuring weighings accurate to 2/100ths of a milligram. Our observations indicate a maximum possible error of 7/100ths of a milligram (due to small additions or losses of oil in the weighing container). This is scarcely significant in the case of ovarian and thyroid weights, permits a maximum possible error of about 6 per cent in anterior pituitary weights, and a maximum possible error of 40 per cent in posterior pituitary weights.

In order to make comparisons between animals having somewhat different body weights the ratio $\frac{\text{gland weight}}{\text{body weight}}$ has been used. This would be strictly usable if gland weight were directly proportional to body weight. Over the range of ages used this is almost, but not quite, true for all the glands examined. The effect of the oestrin injections, however, is measured by the value of $R_C - R_E$ (the last four columns of table 1) where $R_C = \frac{\text{gland weight}}{\text{body weight}}$ of the controls and $R_E = \frac{\text{gland weight}}{\text{body weight}}$ of the injected animals. Where the value of $R_C - R_E$ is positive a relative reduction in gland weight in the experimental animals is indicated; where $R_C - R_E$ is negative a relative increase in gland weight in the experimental animals is indicated.

[1] We are indebted to Dr. E. A. Sharpe of Parke, Davis & Co. for generous amounts of Theelin.

A first inspection of table 1 shows a consistent inhibition of ovarian growth at all ages and for all dosages in the injected animals, *i.e.*, the value

TABLE 1

INJECTED	RAT UNITS PER DAY	BODY WEIGHT	OVARY WEIGHT	ANTERIOR PITUITARY WEIGHT	POSTERIOR PITUITARY WEIGHT	THYROID WEIGHT	$\frac{\text{OVARY WEIGHT}}{\text{BODY WEIGHT}} \times 10^4$	$\frac{\text{A. P. WEIGHT}}{\text{BODY WEIGHT}} \times 10^4$	$\frac{\text{A. P. WEIGHT}}{\text{BODY WEIGHT}} \times 10^4$	$\frac{\text{THYROID WEIGHT}}{\text{BODY WEIGHT}} \times 10^4$	OVARY $R_C - R_E$	ANTERIOR PITUITARY $R_C - R_E$	POSTERIOR PITUITARY $R_C - R_E$	THYROID $R_C - R_E$
days		*grams*	*mgm.*	*mgm.*	*mgm.*	*mgm.*								
5	0	34.4	10.63	1.20	0.21	3.48	3.09	3.49	6.10	9.80				
	1	25.5	7.33	0.85	0.19	2.64	2.87	3.33	7.45	10.35	0.22	0.16	−1.35	−0.55
	2	33.0	9.34	1.28	0.23	3.83	2.83	3.88	6.97	11.61	0.26	−0.39	−0.87	−1.81
	4	28.7	8.27	1.20	0.24	3.27	2.88	4.18	8.36	11.39	0.21	−0.69	−2.26	−1.59
	7	34.8	9.19	1.39	0.18	3.91	2.64	3.99	5.17	11.24	0.45	−0.50	0.93	−1.44
	10	34.5	9.50	1.49	0.26	3.73	2.75	4.32	7.54	10.81	0.34	−0.83	−1.44	−1.01
10	0	45.0	12.11	1.47	0.26	3.74	2.69	3.26	5.77	8.31				
	1	41.8	9.82	1.50	0.24	3.17	2.35	3.59	5.74	7.58	0.34	−0.33	0.03	0.73
	2	35.9	8.82	1.23	0.20	3.41	2.46	3.43	5.57	9.50	0.23	−0.17	0.20	−1.19
	4	40.9	9.16	1.49	0.26	3.79	2.24	3.64	6.36	8.92	0.45	−0.38	−0.59	−0.61
	7	40.4	9.70	1.24	0.23	4.16	2.40	3.07	5.69	10.30	0.29	0.19	0.08	−1.99
	10	40.7	9.82	1.45	0.27	3.78	2.41	3.56	6.63	9.29	0.28	−0.30	−0.86	−0.98
20	0	55.3	11.26	1.65	0.29	3.83	2.04	2.89	5.08	7.41				
	1	62.5	9.38	1.79	0.25	4.60	1.50	2.86	4.00	7.36	0.54	0.03	1.08	0.05
	2	53.6	7.58	1.45	0.23	4.92	1.41	2.71	4.41	9.18	0.63	0.18	0.67	−1.77
	4	58.4	8.87	1.69	0.30	4.61	1.52	2.89	5.14	7.89	0.52	0.00	−0.06	−0.48
	7	53.0	7.57	1.58	0.29	4.45	1.43	2.98	5.47	8.40	0.61	−0.09	−0.39	−0.99
	10	62.3	8.52	1.69	0.31	4.71	1.37	2.71	4.98	7.56	0.67	0.18	0.10	−0.15
30	0	75.8	13.65	1.89	0.35	5.19	1.80	2.49	4.46	6.85				
	1	77.4	8.44	2.31	0.31	5.44	1.09	2.98	4.00	7.03	0.71	−0.49	0.46	−0.18
	2	72.1	9.75	2.00	0.26	4.79	1.35	2.77	3.61	6.64	0.45	−0.28	0.85	0.21
	4	82.2	9.26	2.11	0.35	5.98	1.13	2.57	4.26	7.22	0.67	−0.08	0.20	−0.37
	7	73.2	7.62	2.33	0.30	5.10	1.04	3.18	4.10	6.97	0.76	−0.69	0.36	−0.12
	10	71.6	8.95	2.73	0.33	5.83	1.25	3.81	4.61	8.14	0.55	−1.32	−0.15	−1.29
40	0	97.0	24.58	3.84	0.45	6.03	2.53	3.96	4.62	6.22				
	1	91.5	12.48	2.83	0.33		1.36	3.09	3.61		1.17	0.85	1.01	
	2	97.6	10.70	2.78	0.36		1.10	2.84	3.68		1.43	1.12	0.94	
	4	90.2	11.93	2.86	0.31	6.05	1.32	3.17	3.44	6.71	1.21	0.79	1.18	−0.49
	7	85.8	9.39	2.81	0.28	5.26	1.09	3.28	3.26	6.16	1.44	0.68	1.36	0.06
	10	93.1	12.83	3.38	0.49	6.19	1.38	3.63	5.26	6.65	1.15	0.33	−0.64	−0.43

of $R_C - R_E$ is always positive. Secondly, in all age-groups there is no consistent relation between the extent of the inhibition and the dosage of

hormone injected; one rat unit is as effective as ten rat units in inhibiting ovarian growth. But the extent of the inhibition increases with time, so that after 5 days of injection the ovaries of the experimental animals are 90 per cent as heavy as those of the controls, whereas after 40 days the ovaries of the experimental animals are about 50 per cent as heavy as those of the controls. This inhibition of ovarian weight as measured by $R_C - R_E$ gives the following relation

$$\log (R_C - R_E) = kt + C \tag{1}$$

This is shown graphically in figure 1.

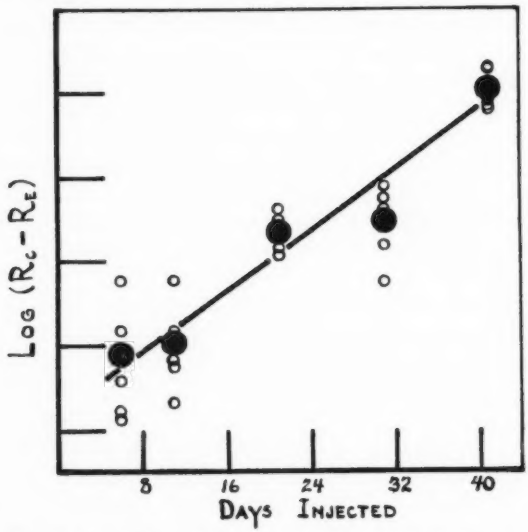


Fig. 1. The logarithm of $R_C - R_E$ for ovarian growth plotted against the number of days after the beginning of oestrin injection. The open circles represent values for each of the five groups killed at the respective times indicated; the closed circles represent the mean values for all the five groups at these times. See text and table 1.

If the value $R_C - R_E$ be taken as a true measure of inhibition, this merely states that oestrin prevents the growth-promoting action of a substance that increases logarithmically with time over the age-range employed. Presumably oestrin injection does not check all growth stimulation, since the ovaries of injected animals do show some growth. If only a single substance is concerned with the stimulation of ovarian growth then we must suppose that the oestrin inhibits the action of only a certain proportion of it. Since the extent of inhibition is independent of dosage (at least over the range of dosages employed in these experiments), it must

follow either that there is only a limited proportion of substance easily "neutralized" by oestrin or that more than one substance is concerned with the promotion of ovarian growth, one of which is "neutralized" by oestrin while the other or others are unaffected.

If the curve of figure 1 be extrapolated to 0 days a small amount of inhibition even at 0 days would be indicated. This is manifestly incorrect. We must conclude accordingly that either our data are somewhat aberrant, or, what is more likely, that the oestrin inhibition takes place rapidly during the first few days of injection. This is in accord with the fact that the inhibition is a function of time and not of dosage, since the implication is that a given limited amount of gonad-stimulating substance is prevented from acting. It seems likely, therefore, that the injection of sufficiently small dosages of oestrin would disclose a relation of effect to dosage.

Experiments reported by Spencer, Gustavson and D'Amour (1930, 1932) and by D'Amour (1931) indicate that there is a relative depression of general growth as well as of ovarian growth in rats receiving continuous oestrin injections. The inference is that there is either a partial inhibition of the effects of the growth hormone of the pituitary as well as inhibition of gonad-stimulating effects, or a general depression of anterior pituitary activity resulting presumably in the lessened production of all anterior pituitary hormones. If a general depression of pituitary activity were occasioned one might expect an inhibition of anterior pituitary growth as well as of ovarian growth, and Kunde, D'Amour, Gustavson, and Carlson (1930, 1931) report a relative decrease in anterior pituitary size in a small series of dogs receiving continuous oestrin injection. In our series of experiments there is no marked inhibition of general growth rate. D'Amour (1931) reports an inhibition of 20 to 25 per cent, whereas the maximum difference in our series is about 11 per cent, and this is not statistically significant. Furthermore, there is no very pronounced indication of reduced pituitary growth, with the possible exception of our forty-day series. The value of $R_C - R_E$ in all our other series is either very slightly positive or negative, indicating either no effect or a slight hyperplasia in the experimental animals. Even in the forty-day series the difference between the control and experimental pituitaries is scarcely significant statistically, and the contrast between the invariable inhibition of ovarian growth in all series and the irregular variations in the anterior pituitary response is, of course, marked.

The indications are, therefore, that there is no general inhibition of gland growth as a result of oestrin injection. This is borne out in the data on posterior pituitary weights. Since these weight data are the least reliable of all in our series no great emphasis is laid upon them. But the general inference is plain: there is no regular variation in the value of $R_C - R_E$, and certainly no regular relation of $R_C - R_E$ either to dosage or to time.

Our data on thyroid weights indicate an increase in weight as a result of oestrin injection, *i.e.*, the value of $R_C - R_E$ is preponderantly negative. This is in accord with the findings of Kunde, D'Amour, Gustavson, and Carlson (1930, 1931). Curiously enough, our data indicate that this weight increase is at a maximum after five days of injection and decreases fairly regularly to a minimum after forty days of injection, as though there is an immediate effect of oestrin injection which continued injection does not sustain. Nor is there any indication of a dosage relation to this effect, with the possible exception of the one-unit injections which appear roughly less effective than the higher dosages.

Discussion. It is perhaps premature to discuss the bearing of these findings on the mechanism of the pituitary-ovary relationship. The following considerations should, however, be kept in mind in proposing an explanation. If ovarian growth is dependent upon the production of a stimulating substance from the anterior pituitary, then our data indicate clearly that only a limited amount of this substance is produced (or "neutralizable" by oestrin) in any interval of time. If the equation (1) given above be taken as correct, the rate of production of this "neutralizable" material may be expressed by the differential expression

$$\frac{d\,(R_C - R_E)}{dt} = k\,(R_C - R_E) \qquad (2)$$

Obviously this expression will hold only for the age-range employed in these experiments, for if it were applied to the whole life of an individual it would imply the production of an almost infinite amount of material late in life, and this is manifestly absurd. Undoubtedly later in life the presumed reaction enters a phase of negative acceleration. Accordingly in any inquiry into the ovary-pituitary mechanism the age of the experimental animals must be a considerable factor.

The findings of these experiments accord well with those of Evans, Meyer, and Simpson (1932) and Reichert, Pencharz, Simpson, Meyer, and Evans (1932). Their experiments establish 1, that the ovary-stimulating action of pregnancy urine extract (prolan) is proportional to dosage only for a limited range of small doses and that a five-fold or even a ten-fold increase of dosage thereafter resulted in no further stimulation of ovary growth; and 2, that prolan was practically ineffective as a stimulant of ovarian growth in hypophysectomized animals. This indicates that prolan activates a given limited amount of pituitary substance concerned with ovary-stimulation. Unfortunately, in the experiments of Evans *et al.* no account was taken of any possible age relationship, but Hill (1932) has shown that ovulation in immature 21 day old mice requires 4 injections of 0.1 cc. pregnancy urine, whereas 6 0.1 cc. injections are required for 14

day old mice. If the action of prolan represents an activation of a pituitary "prohormone" then this indicates a relative increase of that substance with age in young mice.

On this basis also the results of Baum and Pincus (1932) become more understandable, for they found that inhibition of the oestrin effect in ovariectomized mice by pregnancy urine extract was about the same for all dosages of prolan (follutein) above a certain small dosage. In short, only a limited amount of oestrin inhibitor was activable in a given time.

It is hoped that by the development of the technique employed in these experiments some quantitative estimates of the nature of the gonad-stimulating mechanism may be had. Further discussion may, therefore, await a more complete experimental inquiry.

SUMMARY

Continued injection of oestrin (Theelin) into young rats results in an inhibition of ovarian growth and in a certain amount of thyroid hypertrophy, but there is no definite effect on the weight of either the anterior or posterior lobes of the pituitary. The most consistent effect was the inhibition of ovarian growth. The extent of inhibition was not proportionate to the range of dosages employed, but was proportionate to the number of days of injection, such that over the age-range employed the extent of inhibition increased logarithmically with time.

BIBLIOGRAPHY

Baum, O. S. and G. Pincus. 1932. This Journal, cii, 241.

Crozier, W. J. and G. Pincus. 1931. Journ. Gen. Physiol., xv, 201.

D'Amour, F. E. 1931. Journ. Biol. Chem., xcii, lxxxv.

Evans, H. M., K. Meyer and E. Simpson. 1932. This Journal, c, 141.

Hill, M. 1932. Journ. Physiol., lxxv, 44.

Kunde, M. M., F. E. D'Amour, R. G. Gustavson and A. J. Carlson. 1930. Proc. Soc. Exp. Biol. and Med., xxviii, 122.

Kunde, M. M., F. E. D'Amour, A. J. Carlson and R. G. Gustavson. 1930. This Journal, xcv, 630.

Reichert, F. L., R. I. Pencharz, M. E. Simpson, K. Meyer and H. M. Evans. 1932. This Journal, c, 157.

Spencer, J., R. G. Gustavson and F. E. D'Amour. 1931. Proc. Soc. Exp. Biol. and Med., xxviii, 500.

Spencer, J., F. E. D'Amour and R. G. Gustavson. 1932. Amer. Journ. Anat., l, 129.

THE FAILURE OF SYMPATHETIC EXTIRPATION IN THE RAT TO AFFECT THE BASAL METABOLISM OR THE CALORIGENIC ACTION OF ADRENALINE

M. O. LEE AND Z. M. BACQ[1]

From the Memorial Foundation for Neuro-Endocrine Research, Harvard Medical School, Boston

Received for publication November 4, 1932

It is firmly established that the sympathetic nervous system and its synergist, the adrenal medulla, play important rôles in most body activities which are accompanied by energy expenditures above the basal level. Whether these factors are at all significant in determining and regulating the *basal* level of heat production—and of heat loss—is not clear. The marked calorigenic action of injected adrenaline both during rest and exercise, is well known. In certain conditions, such as hyperthyroidism, in which the basal heat production is greater than normal, the sympathetic system is chronically hyperactive or hyperirritable. On the other hand, during sleep, when the heat production is depressed below the "basal" level, the activity of the sympathetics is presumably depressed like that of the rest of the nervous system. The fact that the metabolic rate is not constant from day to day within the limits one could reasonably set to allow for varying slight degrees of muscular tonus and the unavoidable errors of careful technic in measurement, points to the delicacy of the adjustment.

These facts and others that Cannon (1) has summarized, suggest that the heat production of the body under those conditions which for practical purposes are designated as "basal" represents either a homeostatic equilibrium between factors tending to increase and to decrease catabolism or that relatively constant nervous or endocrine factors are acting tonically in maintaining it above a minimal level. In either case the sympathico-adrenal system is under suspicion as a regulatory mechanism. On this theory, interference with the mechanism, by the removal of much of the sympathetic system, by disconnecting the remnants from the central nervous system and by denervation of the adrenal medulla might be expected to reduce the basal heat production of the animal.

Experimental evidence on these points, except for that of Cannon, New-

[1] Fellow of the C. R. B. Educational Foundation.

ton, Bright, Menkin and Moore (2) in the cat, is lacking or susceptible of equivocal interpretation. Bilateral adrenal extirpation causes a prompt fall in the basal metabolic rate, which may continue until death (3, 4, 5, and observations of our own in rats). But this experiment does not distinguish the rôles of cortex and medulla. There is some evidence of a direct effect of the cortex on metabolism, and of an indirect effect that follows the syndrome of cachexia, emaciation and the inability to control heat loss, that develops in acute insufficiency. Aub, Forman and Bright (3) attempted to determine the relative responsibilities of cortex and medulla for the prompt fall in the basal metabolic rate after adrenalectomy and inclined to the conclusion that the loss of the medulla is responsible. Their evidence, however, is far from satisfactory. They were unable to remove all of the medulla and still leave enough cortex to keep the animal alive. The extracts of cortex which they (6) injected were of unproved potency. They (3) reported results on one animal in which the right adrenal gland was removed and the left denervated by section of the splanchnics. This animal showed an 8 per cent gain in weight and a 19 per cent reduction in metabolism, which, unlike the fall in doubly adrenalectomized cats, became established only after several weeks.

The results obtained by Cannon *et al.* (2) on sympathectomized cats afford more satisfactory evidence. These workers noted a temporary decrease (maximum = 14 per cent) in the metabolic rate after cervical sympathectomy in four cats, and a smaller depression after the abdominal and thoracic sympathetics were removed. The metabolic rates fell slowly and after several months recovered to near the original levels. The present paper reports a series of determinations of the basal metabolic rates and the calorigenic effects of several dosages of adrenaline in albino rats before and after the removal of portions of the sympathetic system and the denervation of one adrenal and removal of the other.

METHODS AND RESULTS. The rats were sympathectomized by the technic previously described by Bacq (7). At the first operation both abdominal ganglionated chains were removed from the second lumbar ganglia to the pelvic brim; the right adrenal gland was completely removed; the left adrenal was denervated by destroying all of the splanchnic branches that could be found in the mesentery, by removal of the adrenal ganglion and by scraping and cleaning the stalk formed by the adrenal artery and vein; in three of the rats the superior mesenteric plexus was also removed. At the second operation, usually about four weeks later, both stellate ganglia were extirpated and from 3 to 15 mm. of each thoracic chain. At the third operation the left splanchnic nerve and the remainder of the left thoracic chain were ablated.

Eight rats which recovered satisfactorily after one or more stages of sympathetic removal are included in the series. Four of these either died

after the stellate ganglia and upper thoracic chains were removed, or showed signs of infection which rendered them unsuitable for metabolic experiments. Only two rats of the series recovered properly after all three operations. These two remained in excellent health, gained in weight, and gave many metabolic rate determinations which were technically satisfactory (i.e., with minimal or no activity, and the apparatus working perfectly). Five of the rats had served as controls in other metabolism experiments, and a long series of determinations had fixed their normal levels with a high degree of certainty. In the other three rats, five or six determinations of the basal metabolic rates and of the calorigenic effects of adrenaline were

TABLE 1

Mean basal metabolic rates before and after successive stages of sympathectomy

RAT NUMBER	CALORIES PER DAY PER SQUARE METER, AND STANDARD DEVIATION			
	Before sympathectomy	I. Abdominal sympathectomy	II. Stellates and upper thoracic chains out	III. Lower thoracic chain out
I	**8** 781 ±34	**7** 772 ±38		
II	**12** 772 ±35	**8** 791 ±43		
III	**5** 809 ±31	**7** 838 ±25		
IV	**6** 817 ±30	**7** 806 ±27		
V	**10** 798 ±44	**6** 809 ±33	**7** 786 ±36	
VI	**5** 775 ±37	**9** 777 ±46	**8** 775 ±36	
VII	**9** 795 ±36	**8** 787 ±42	**11** 783 ±33	**8** 794 ±29
VIII	**15** 831 ±35	**9** 811 ±33	**8** 826 ±37	**6** 831 ±41
Mean,* all rats	797 ±5†	799 ±5†		
Mean,* rats V, VI, VII, VIII	800 ±9†	796 ±6†	793 ±9†	

The figures in boldfaced type are the numbers of determinations (different days), giving the following mean and standard deviation from that mean.

* Not weighted.

† Probable error of the mean.

made before the first operation. All of the rats were adult males, ranging in weight from 230 to 410 grams.

After each stage of sympathetic removal from 6 to 11 metabolic rate determinations were made on each rat, beginning a few days after the operation and continuing two or three times weekly. An oxygen consumption method was used, with a closed circuit apparatus. All determinations were made at 29.5°C., after the animal had fasted from 18 to 24 hours. A few respiratory quotients were determined, all after abdominal sympathectomy. These ranged from 0.70 to 0.75, which is about the same as in the normal rat.

A number of measurements of the calorigenic effect of adrenaline were

likewise made before the first operation and following each stage of sympathectomy. The routine procedure on any day was first to determine the basal rate, then to administer a dose of adrenaline and follow the metabolism until an approximate calculation showed it had returned to the basal level. Usually a second dose was then given and the effect similarly followed. Six dosages of adrenaline were used, 0.0037, 0.0075, 0.010, 0.015, 0.020, and 0.030 mgm. per kgm. of body weight. The injections were all made intraperitoneally, because absorption by the subcutaneous route is very slow. Even when administered intraperitoneally there was sometimes no effect for an hour or so, and in four instances the experiment was terminated after three or four hours without any effect having been produced. For the most part, the metabolic effect was apparent within 15 to 30 minutes, as shown by graphic records, and was all over in two to

TABLE 2

Mean calorigenic effects of adrenaline before and after successive stages of sympathectomy

DOSAGE	EXCESS OXYGEN USED			
	Before sympathectomy, 8 rats	I. Abdominal sympathectomy, 8 rats	II. Stellates and upper thoracic chains out, 4 rats	III. Lower thoracic chain out, 2 rats
mgm./kgm.	*cc. O_2*	*cc. O_2*	*cc. O_2*	*cc. O_2*
Placebo	**4** 28	**3** 5	**4** 18	
0.0037	**3** 25	**4** 35		
0.0075	**9** 43	**10** 69	**5** 55	**6** 61
0.010	**6** 77	**5** 48	**5** 65	**3** 50
0.015	**6** 76	**7** 87	**6** 65	**4** 71
0.020	**6** 92	**7** 99	**9** 83	**5** 92
0.030	**2** 102	**2** 131		

The figures in bold face type are the numbers of determinations.

three hours. Restlessness and gross body movements were not increased by the adrenaline.

In calculations, the basal rate measured just before an adrenaline injection, was used, rather than the average level. The calorigenic effect is expressed in table 2 as the total excess oxygen consumed after adrenaline until the return to the basal level. The accuracy of the estimation of the adrenaline effects was of course not as great as that of the basal rate determinations. The animals had to be removed from their jars for the injections, and although a rest period of 8 to 15 minutes was allowed before starting the measurements, the effects of the handling had perhaps not entirely worn off, or some of the adrenaline effect may have been missed. About as often as not the basal rate reached after the adrenaline effect had worn off was somewhat lower than the preliminary basal rate. It was impossible to estimate the return to the basal rate closer than within 30

minutes or so, and if the rate during that went time much below the original basal, an indeterminate error was introduced into the calculations. On the other hand, the effect was occasionally slow in appearing and in passing off and the experiment had to be ended before the original level was quite reached. These factors probably account for the comparatively large variations in the calorigienic responses, even to the same dosage.

Both the data for the basal metabolic levels and those for the effect of adrenaline were unselected, except on technical considerations. That is, in relatively few instances continued restlessness and movements of the rats necessitated the elimination of the day's determinations. Seven determinations of the calorigenic response to adrenaline, in which the experiment had to be ended before any effect was produced or while the slow response was obviously at its height, were not included in the average.

Discussion. The results definitely fail to show an effect on the basal level of heat production after the removal of any part of the sympathetic systems and inactivation of the adrenal medulla. Examination of the serial determinations did not reveal a transitory effect with a return to normal after any operation, contrary to results on the cat (2). While it is true that the operative procedures were not identical with those for the cat and the cervical ganglia and nerves were not removed, the extirpation of the upper thoracic chains and the stellate ganglia should have completely isolated these cervical ganglia from the rest of the nervous system. The variability of the basal rate from day to day, as measured by the standard deviations from the means was not changed enough to indicate either a greater or less stability of the level after sympathectomy. Changes in weight in the animals were entirely insufficient to make any changes in metabolism. The means of the total oxygen consumption per day, or per day per 100 grams weight, were no more suggestive of a difference after sympathectomy than were those of the heat production expressed in terms of body surface, which is calculated from both body weight and length (8).

Likewise, the results leave no doubt as to the persistence of the calorigenic action of adrenaline. This latter finding is not surprising and confirms previous conclusions that the locus of the calorigenic, like that of the other actions of the hormone, is peripheral, involving the tissues. Neither the ganglion centers of the chains with their central nervous connections nor the postganglionic fibers are necessary for this action.

The only explanation of the difference in metabolic results in the cat and rat that seems worth offering at present is that of species difference and the possible influence of a carefully regulated environment and heredity upon the adaptive mechanisms of rats. These animals (Wistar strain) for many generations have spent their lives in small cages with food *ad libitum* and a temperature variation of probably not more than 15°C. They are gentled from infancy and the greatest vicissitudes they have ever to meet are

occasional infections and a few fights. No similar conditions exist in most other species of mammals. The most comparable among human beings might be found only in institutions for mental defectives or for certain types of insanity.

Benedict and Petrìk (9) have shown that the metabolic rate of wild Norway rats (under strictly basal conditions) is considerably higher than that of the tame albino variety. Watson (10) found that the weight of the adrenal glands was more than twice as great in wild Norway rats as in the tame albino. These observations suggest that sympathectomy in the wild variety might give noticeable effects. The data of the present paper indicate that in the normal laboratory albino, basal and minimal metabolism may be synonymous terms and regulation by the sympathico-adrenal system an insignificant factor. These data, of course, have no cogency in relation to the adaptive significance of the system in other than basal conditions.

SUMMARY

In eight albino rats the abdominal sympathetic chains and much of the thoracic chains including the stellate ganglia were removed; the left adrenal was denervated and the right removed. Analysis of 179 determinations of the basal metabolic rates in these animals failed to show any change in the metabolic levels after any stage of sympathectomy. The calorigenic responses to injected adrenaline, in dosages of 0.0037 to 0.03 mgm. per kgm., were likewise unaltered.

BIBLIOGRAPHY

(1) Cannon, W. B. Physiol. Rev., 1929, ix, 399.
(2) Cannon, W. B., H. F. Newton, E. M. Bright, V. Menkin and R. M. Moore. This Journal, 1929, lxxxix, 84.
(3) Aub, J. C., J. Forman and E. M. Bright. This Journal, 1922, lxi, 326.
(4) Barlow, O. W. This Journal, 1924, lxx, 453.
(5) Demaría Massey, C. Rev. Soc. argent. de biol., 1927, iii, 5.
(6) Aub, J. C., E. M. Bright and J. Forman. This Journal., 1922, lxi, 349.
(7) Bacq, Z. M. This Journal, 1932, xcix, 444.
(8) Lee, M. O. and E. Clark. This Journal, 1929, lxxxix, 24.
(9) Benedict, F. G. and J. M. Petrìk. This Journal, 1930, xciv, 662.
(10) Watson, C. Journ. Physiol., 1907, xxxv, 230.

THE TIME AT WHICH CASEIN BEGINS TO BE FORMED IN THE BREAST DURING PREGNANCY

ORAN I. CUTLER AND JULIAN H. LEWIS

From the Department of Pathology of the University of Chicago and the Otho S. A. Sprague Memorial Institute

Received for publication November 2, 1932

There have been numerous observations of gross and histological changes occurring in the breasts of mammals while pregnant. The chemical processes which take place in the breast during the progress of pregnancy have been less completely investigated. That lactose begins to appear in the blood and urine of a mother some time before the close of pregnancy is well known (1). It would be of interest to determine just when each of the organic substances found in milk begin to be formed in the breast.

One of the materials which is most characteristic of milk is casein. This protein seems to be in the milk of all mammals. In the investigations described in this paper, udders of cattle have been tested for the presence of casein. For the purpose of making this study, a series of breasts from freshly killed cattle was obtained from a packing house. An endeavor was made to take udders from heifers which either were not pregnant or were in their first pregnancy. When a fetus was present its size was noted in each case in order to determine the length of time the animal had been pregnant. Old cows were avoided because of the possibility that they might have been lactating at the time they became pregnant.

Each breast was ground fine and extracted with distilled water. The extract was brought to 100°C. to precipitate heat-coagulable proteins present. The fluid remaining after this procedure was separated from the coagulum. The fluid was acidified to a pH of 4.6 to precipitate casein which might be present, together with any other proteins having approximately the same isoelectric point. The precipitate obtained in this way was washed with distilled water and extracted over night with 70 per cent alcohol to remove alcohol-soluble proteins. The precipitate was then washed with 95 per cent alcohol, and with ether and then dried.

The precipitate remaining after the extraction with alcohol was suspended in normal saline, three milligrams of precipitate being added to each cubic centimeter of saline. One-tenth normal NaOH was added until a pH of approximately 7 was reached, at which point most of the precipitate dissolved. In order to determine whether or not casein was

Complement fixation tests for casein in breasts

	ANTI-CASEIN SERUM					CONTROLS—NO ANTISERUM				
	Volume of antigen used									
	0.5 cc.	0.4 cc.	0.3 cc.	0.2 cc.	0.1 cc.	0.5 cc.	0.4 cc.	0.3 cc.	0.2 cc.	0.1 cc.
1. Non-pregnant heifer	0	++	+++	++++	++++	0	++	+++	++++	++++
2. Non-pregnant heifer	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
3. Corpus luteum—no fetus	+	++++	++++	++++	++++	+	++++	++++	++++	++++
4. Fetus 0.5 cm.—3 weeks	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5. Fetus 4 cm.—6 weeks	+	+	+++	++++	++++	+	++	+++	++++	++++
6. Fetus 7 cm.—9 weeks	+	++	++++	++++	++++	+	+++	++++	++++	++++
7. Fetus 14 cm.—12 weeks	++	+++	++++	++++	++++	++	+++	++++	++++	++++
8. Fetus 28 cm.—17 weeks	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
9. Fetus 29 cm.—17 weeks	0	0	+	++++	++++	0	0	+	++++	++++
10. Fetus 33 cm.—18 weeks	0	++	+++	++++	++++	0	++	++++	++++	++++
11. Fetus 36 cm.—19 weeks	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
12. Fetus 55 cm.—25 weeks	0	0	0	0	0	++++	++++	++++	++++	++++
13. Fetus 72 cm.—29 weeks	0	0	0	++	+++	++++	++++	++++	++++	++++
14. Fetus 75 cm.—31 weeks	0	0	0	++	++++	++++	++++	++++	++++	++++
15. Fetus 85 cm.—36 weeks	0	0	0	++	++++	++++	++++	++++	++++	++++
16. Fetus 92 cm.—40 weeks	0	0	0	0	+++	++++	++++	++++	++++	++++

present in this solution, complement fixation tests were performed. The above solutions of protein from udders were used as antigens in volumes of 0.5, 0.4, 0.3, 0.2 and 0.1 cc., the total volume of each being made 0.5 with saline. Controls of antigen without antiserum were made in each experiment. In setting up these tests serum from a rabbit which had received a series of injections of highly purified casein was used. Previous tests had shown that when a complement fixation technique similar to that described here was employed, 0.25 cc. of this serum used in a one to 10 dilution would fix complement in the presence of 0.004 mgm. of casein. In performing the tests for casein in the solutions of proteins from cows udders the inactivated anticasein serum was used in the same amounts as in the preliminary titrations of the serum mentioned above. Two units of complement in a volume of 0.25 cc. were used. As an indicator of fixation 0.25 cc. of 2.5 per cent suspension of washed and sensitized red cells was added to each tube. The total volume in each tube was 1.25 cc. The primary and secondary incubation was in each case for one hour in the water bath at 37°C. The results were read immediately as 0, +, ++,+++, ++++ according to the degree of hemolysis.

As is shown in the appended table, a number of preparations obtained from udders by the method outlined above appeared to contain casein. The method of acid precipitation and resolution in alkali made possible the concentration of any casein present in rather small volumes of fluid. That other proteins than casein were present in the precipitates is shown by the fact that some of them, when used as antigens, failed to cause complement fixation in the presence of anti-casein serum.

Discussion. The results of complement fixation tests for the presence of casein in the udders studied indicate that casein can be found in udders of cattle after the middle of pregnancy. The normal duration of pregnancy in the cow is nearly the same as in woman. During the first half of pregnancy the breast rather slowly enlarges. Before the middle of pregnancy is reached, a quantity of clear pale yellow fluid begins to appear in the ducts of the udder (2). By the time prgenancy is half completed, this fluid begins to be turbid and gradually takes on the appearance of milk. The turbidity is noticeable before casein can be found by complement fixation tests. Migliavacca (3) has described certain nongranules which he has found in breast epithelium during the last half of pregnancy. These granules are found in the epithelial cells and later extruded into the lumina of the glands. Migliavacca believes that these granules may have some relationship to the colostrum corpuscles.

The series of tests which have been made is too small to determine more than approximately the date at which casein may be found in the udder during pregnancy. When a larger number of breasts from pregnant animals have been examined for the presence of casein, the time when it

begins to be formed can be stated more exactly. In the animals studied, casein was found after the middle of pregnancy, between the 20th and 25th week. It might be found to appear a little later or earlier in other cows. Also, the date at which casein begins to be formed may not be the same in other animals and in woman. A study of the effect of hormones of endocrine glands on the breast in relation to the formation of casein would be of decided interest. A method of assay of such materials as extracts of the hypophysis might be provided by such an investigation.

The question has arisen as to whether casein, like lactose, is present in the blood of lactating animals. In an attempt to answer this question a liberal quantity of blood from a lactating cow was treated in similar manner to the extracts of udders described above. The results of the tests for casein which have been made, so far, are negative. Modification of the process employed in treating the blood to extract casein may make possible the demonstration of this protein in the blood of lactating animals.

CONCLUSIONS

A series of 16 udders of cattle at various stages of their first pregnancy was tested for the presence of casein by the method of complement fixation, using a casein antiserum.

Casein was demonstrable in all udders after the 24th week of pregnancy (estimated) and in no udder of heifers pregnant less than 20 weeks.

BIBLIOGRAPHY

(1) WOLKINS, O. Journ. Biol. Chem., 1928, lxxx, 33.
(2) HAMMOND, J. The physiology of reproduction in the cow. Cambridge Univ. Press, 1927, p. 117, 157, 168–178.
(3) MIGLIAVACCA, A. Folia Gyn., 1930, xxvii, 285.

ABNORMAL GROWTH OF WATTLES AND TESTES OF COCKERELS AFTER REMOVAL OF COMBS[1]

G. DAVIS BUCKNER, W. M. INSKO, JR. AND J. HOLMES MARTIN

From the Kentucky Agricultural Experiment Station, Lexington, Ky.

Received for publication November 9, 1932

Smith (1) has observed that the increase in the size of hen's combs at the commencement of egg laying periods was due to a fatty infiltration of the central connective tissue core and that the subsequent decrease was due to the abstraction of fat. The cock's comb, consisting mainly of fibrous connective tissue and containing only a comparatively small quantity of fat in the central core, does not vary in size as the hen's comb does.

Womack, Koch, Domm and Juhn (2) have shown that light retards or lowers the comb growth response in Brown Leghorn capons to a given dose of testicular hormone and discuss unpublished data of Dr. L. V. Domm concerning the seasonal variations in the sizes of combs of normal cocks and poulards which are possibly influenced by light.

In a previous communication (3) the authors presented data which showed that cockerels raised in battery brooders, from which direct sunlight was excluded, developed abnormally large combs and smaller testes than those raised in a colony brooder, with access to a grass range and receiving direct sunlight. At the time these measurements and observations were made, it was noted also that the wattles of the cockerels raised in the battery brooders were larger than the wattles of those raised in the colony brooder under the conditions previously mentioned. The cause of the production of large combs and wattles and small testes in cockerels raised in confinement and in the absence of direct sunlight is unknown, yet it seems possible that these conditions are connected with the exclusion of direct sunlight and that the cockerels elaborated combs and wattles with larger surfaces to compensate for the decreased quantity of solar radiation received.

Having obtained these results it seemed of interest and importance to determine what effect the removal of the combs at an early age would have on the subsequent growth and development of the wattles and testes, when cockerels are raised in confinement in battery brooders, in the absence

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station, and is published by permission of the Director.

of direct sunlight. With this end in view, 110 White Leghorn cockerels which came from the same parent stock and were hatched the same day, February 13, 1932, were placed in a wire-floored, hot-water-heated battery brooder of the usual type, so situated in a small animal house as to receive no direct sunlight. These chicks were fed a ration which had been found experimentally to supply all the necessary food factors for the growing chick, similar to that used in our previous experiment (3). It consisted of wheat bran 25, wheat middlings 25, ground yellow corn 25, meat scrap (50 per cent protein) 12, dried buttermilk 10, salt 1 and steamed bone meal 5 parts. Enough of a commercial cod liver oil concentrate was added to make 0.125 per cent of the mash. The mash and a grain mixture consisting of equal parts of cracked yellow corn and wheat were fed *ad libitum* in hoppers. On March 12, 1932, at the age of 4 weeks, 16 cockerels of approximately the same weight were selected and separated into 3 lots according to the following conditions: lots 1 and 2 contained 6 and 5 birds, respectively, whose combs were normal, approximately the same size and erect; lot 3 contained 5 cockerels whose combs were approximately the same size as those in the other two lots, but instead of being erect, drooped to one side.

The combs of the ten cockerels in lots 2 and 3 were cut off evenly with the top of the head, with shears, and after the bleeding had ceased all 16 cockerels were placed together in the battery brooder. They received the ration previously described and existed under identical conditions until they were 14 weeks of age. At this time, May 21, 1932, the cockerels were weighed individually and killed. The combs and wattles of the cockerels in lot 1 and the wattles of the cockerels in lots 2 and 3 were cut off close to the head, immediately weighed and the surface areas of each determined with a planimeter, estimating the surface as twice the reading. The cockerels were then cut open and the testes dissected out and immediately weighed. A microscopical examination of the enlarged wattles of the cockerels in lots 2 and 3 showed a simple physiological hyperplasia and no excessive fatty infiltration.[2]

At the time the cockerels were killed, it was noted that the shanks of practically all those in lots 2 and 3 were a paler yellow color than those of the cockerels in lot 1 whose combs were not removed.

In the table it will be seen that the cockerels were practically the same size in all three lots, as shown by the weights.

In lots 2 and 3, in which the combs had been removed, the wattles were heavier and larger than those of the cockerels in lot 1, which did not have their combs removed, and the testes were approximately twice as heavy, under the corresponding condition.

[2] Microscopical examination made by Dr. D. J. Healy of the Department of Animal Pathology, Kentucky Agricultural Experiment Station.

Bissonnette (4) discusses Rowan's experiments as described in his book, *The Riddle of Migration*, in which it was indicated that increasing periods of wakefulness or of muscular exercise by day are direct causes of the spring increase in the size and activity of the reproductive glands of juncos and presumably of other birds such as poultry, and does not credit the increas-

TABLE 1

Measurements of the 14-weeks-old white leghorn cockerels raised under the experimental conditions described

COCK NUMBER	WEIGHT OF COCK	WEIGHT OF COMB	AREA OF COMB	WEIGHT OF 2 WATTLES	AREA OF 2 WATTLES	WEIGHT OF 2 TESTES
	Lot 1. Erect combs, not removed					
	grams	*grams*	*sq. cm.*	*grams*	*sq. cm.*	*grams*
506	1,100	13.0	51.2	4.4	22.4	0.8
561	1,117	32.3	94.4	2.0	23.6	1.2
578	1,190	34.9	112.4	2.2	27.6	3.2
647	1,260	14.9	83.0	2.6	27.6	5.2
672	1,180	19.2	66.4	3.0	26.8	2.2
746	1,067	24.2	78.8	2.4	23.6	4.4
Average..	1,152	23.1	81.0	2.8	25.3	2.8
	Lot 2. Erect combs, removed					
532	1,114			4.8	48.8	6.2
593	1,230			3.0	66.4	5.2
661	1,160			4.8	58.0	5.0
715	1,110			6.2	65.6	7.0
738	1,168			5.4	49.6	9.0
Average..	1,156			5.0	57.7	6.5
	Lot 3. Drooping combs, removed					
576	1,245			3.6	28.4	8.4
594	900			2.8	33.6	0.8
677	950			2.8	44.0	4.6
707	1,140			5.6	63.2	3.0
733	1,350			3.6	36.4	4.9
Average..	1,117			3.7	41.1	4.3

ing periods of exposure to light radiation as being a contributing factor. He also discusses his own work on the starling, which indicates that the size and activity of the testes are conditioned by the daily period, intensity and wave length of light to which the birds are exposed, and that increasing the exercise periods does not induce sex-gland activity.

In our experiment the increase in the size of the wattles and the testes is

not the result of either exercise or direct solar radiation. The increase in the size of the wattles is not caused by fatty infiltration but is probably compensatory for the removal of the combs and diminished total surface area which apparently is sensitive to the direct radiant energy of the sun. The cause of the increase in the size of the testes of the cockerels whose combs were removed is unknown. However, these results show that when raised in confinement in battery brooders from which direct sunlight was excluded the cockerels whose combs had been removed when they were four weeks of age developed larger wattles and testes than those raised under similar conditions whose combs had not been removed.

BIBLIOGRAPHY

(1) Smith, G. Quart. Journ. Micr. Sci., 1911, lvii, 45.
(2) Womack, E. B., F. C. Koch, L. V. Domm and M. Juhn. Journ. Pharm. Exper. Therap., 1931, xl, 173.
(3) Buckner, G. D., W. M. Insko, Jr. and J. H. Martin. This Journal, 1932, cii, 271.
(4) Bissonnette, T. H. Science, 1932, lxxvi, 253.

A COMPARISON OF THE TIME RELATIONS OF MUSCLES SUPPLIED BY NORMAL AND BY REGENERATED NERVES

DONALD H. BARRON

From the Osborn Zoölogical Laboratory, Yale University

Received for publication October 27, 1932

Since Biedermann (1895) first suggested that the wide variation in the diameter of nerve fibers was correlated with their physiological function, numerous studies have been reported which extend this suggestion and appear to bear out Biedermann's statement that differences of excitatory effect are not due solely to differences in the terminal end organs.

Lapicque and Legendre (1913) first pointed out that the chronaxie of various nerve fibers in the frog varied with their diameter. These results were later confirmed by Lapicque and the Desoilles (1927) and Kreindler (1927).

The relations between the diameter of an axon and the speed of its action potential have been demonstrated by Gasser and Erlanger (1926) and Lapicque (1926). Lapicque states that the speed of the action potential is directly related to the diameter of the axon and its chronaxie; the action potential travels one centimeter in one chronaxie. The rising phase of the action potential also varies with the diameter of the axon and is three chronaxies in duration. Mathews (1929) found that the duration of the action potential also varies with the diameter of the axon—the larger the axon, the shorter the duration of the action potential.

Lapicque in his review (1926) has shown that the chronaxie of a motor nerve and the muscle fibers that it supplies normally have the same chronaxie (isochronism). This state of isochronism is necessary in order that stimuli arising in the motor nerve may excite the muscle fibers to contraction. If a state of heterochronism exists between the muscle and its motor nerve, stimuli arising in the nerve do not excite the muscle to contraction. These facts suggest that a relationship exists on the basis of the findings stated above between the chronaxie of a muscle and the diameter of the motor axons supplying it. Indeed this suggestion is not without further evidence supporting it. Carlson (1905) pointed out that the speed of the impulse travelling in a motor axon was related to the duration of the contraction of the muscle which it supplied. The more rapidly the motor axons conducted, the shorter the duration of the contraction of the muscle. Since the speed of the nervous impulses has been shown to be related to the

diameter of the axon, it is interesting that Hay (1901) found that the axons supplying pale muscle are about 5μ larger in diameter than those supplying red muscle. It has been repeatedly shown by Fulton (1926), Cash (1880), Kronecker and Stirling (1878) that the duration of the contraction of red muscle markedly exceeds that of pale fibers.

These facts are supported by other evidence. Starling (1930) states that the duration of the contraction of a muscle is related to its chronaxie. This observation supports and extends the other statements made above which strongly urge that a definite relationship, both anatomical and physiological, exists between a muscle and the motor nerve supplying it.

These facts and the generalizations drawn from them have been obtained by a comparison of data selected from varying sources. The diameter of the motor axons recorded appears in each case to be taken at some point in the course of the nerve and does not refer specifically to the diameter of the intramuscular axons. These generalizations could be definitely checked, provided the diameter of the axons of a peripheral nerve could be altered and allowed to supply the same muscle. The relationship between the diameter of the motor nerve and the chronaxie of the muscle it supplies, as well as the duration of the contraction, and the tension developed could be compared with the normal for that individual muscle.

The diameter of the axons supplying a given muscle can be altered experimentally by sectioning the nerve and allowing it to regenerate, for it has been observed that the regenerated axons of a spinal nerve are of a smaller diameter than those which originally made up the trunk (Greenman, 1913; Barron, 1932). Greenman crushed the peroneal nerve of albino rats of about 135 grams in weight. The sectional area of the ten largest fibers on the proximal side of the lesion was 55.8 sq. micra or 15 per cent less than the area of the fibers of the control. The sectional area of the ten largest regenerated fibers on the distal side of the lesion was 29.9 sq. micra, or 54 per cent less than the area of the fibers of the control nerve. Further, in the test animals in which both the control and operated nerves were diminished in total area, the axis sheath relation was such that in all three localities the area of the axis cylinder was relatively less than in the fibers of the normal animal.

These results have been confirmed (Barron, 1932) and extended to include observations on the median, ulnar, and the posterior tibial nerves of the albino rats. The diameter of regenerated axons in these nerve trunks holds no relation to the diameter of the axons which originally made up the nerve bundle. The largest axons (less than 1 per cent in the regenerated nerve trunk) were only 3.4 mu in diameter. The majority were about 1 micron. The original nerve trunks contained axons up to 11.2 mu and the bulk of the fibers were over 3.4 mu in diameter.

This method of sectioning or crushing a peripheral nerve and allowing it

to regenerate into the muscle groups it normally innervated was used to obtain muscles supplied by axons of a smaller diameter than those by which they were originally innervated.

MATERIALS AND METHODS. Young male albino rats weighing between 175 and 200 grams were selected for the experiments. They were anesthetized with *sodium amytal* (Nicholas and Barron, 1932). The median and ulnar nerves were then exposed and crushed in the mid brachial region. Faradic stimulation of the nerves above this point, following the crushing, failed in all cases to cause contraction of any of the flexor muscles of the fore limb.

The animals were placed in large cages during recovery. The muscle groups supplied by the regenerated median and ulnar nerves were observed to react about forty to fifty days after the operation. The animals then were anesthetized again with *sodium amytal*, and the physiological characteristics of the muscles supplied by both the regenerated and the control nerves were tested.

The chronaxie[1] of the muscle stimulated through the nerve was determined first. The median nerve was placed on Ag-AgCl electrodes of the Lapicque type and covered. The preparation was allowed to remain between twenty and thirty minutes before the rheobase was determined. The current was supplied by a high voltage "B" battery which was tested before each determination. The rheocord was also of the type recommended by Lapicque. A Keith-Lucas pendulum was used to measure the duration of the stimulus. The influence of the *sodium amytal* anesthesia upon the chronaxie was controlled by determinations on the same animals under ether. The results were the same for both anesthetics. *Sodium amytal* proved more convenient to use and was more easily controlled.

The flexor muscles of a single segment of a limb all have the same chronaxie, as demonstrated by Bourguignon (1923) therefore the slightest twitching of any of the flexor muscle fibers was used as an index in the determination of both the chronaxie and the rheobase.

The isotonic contractions of the muscles were taken a few days later. The animal was again anesthetized with *sodium amytal*. The tendon of the M. flexor digitorum profundus was dissected free and fastened to a muscle lever weighted with ten grams. The radius was exposed by dissection and fastened rigidly with a clamp to an upright which supported the muscle lever. The skin was closed about the clamp to prevent cooling of the muscles. The median and ulnar nerves were exposed by a small incision, cut and placed on silver wire electrodes. The circulation of the flexor muscle group was not interfered with and the preparation lasted until the animal recovered from the anesthesia.

[1] I wish to thank Prof. J. G. Dusser de Barenne of the Department of Physiology, Yale University, for so kindly affording me the facilities with which to make these determinations.

The isometric contractions were recorded by means of a torsion wire myograph built after the description given by Fulton (1926). The myograph was rigidly braced to prevent vibration. The animal was prepared as described for the isotonic twitches except that the limb was held more rigidly by clamping the humerus to the bar supporting the myograph. The tendon of the M. flexor digitorum profundus was attached to the myograph with a light steel wire.

The records of the chronaxie, isotonic and isometric contractions were compared to similar records made from control animals of the same weight and prepared in the same manner.

Immediately after the records of the muscle contractions had been obtained, the median and ulnar nerves were dissected free. Especial care was

TABLE 1

The chronaxie of the M. flexor digitorum profundus in the albino rat when supplied by normal and by regenerated axons

ANIMAL NUMBER	ANIMAL	RHEOBASE	CHRONAXIE
		volt	*sigma*
5-11-32-1	Test	0.1	0.08–0.12
5-11-32-2	Control	0.12	0.10–0.12
6-17-32-1	Test	0.1	0.06–0.10
6-15-32-1	Control	0.15	0.08–0.12
6-26-32-1	Test	0.09	0.06–0.12
5-24-32-1	Control	0.12	0.06–0.09
5-24-32-2	Test	0.12	0.06–0.12
5-26-32-1	Test	0.10	0.08–0.12
5-26-32-2	Control	0.12	0.06–0.10
5-28-32-1	Control	0.11	0.06–0.12

taken to preserve the branches which ran to the flexor muscles of the digits and wrist. These pieces of nerve were fixed in 1 per cent osmic acid for twelve hours, dehydrated, imbedded in paraffin and sectioned serially.

Similar preparations were made from control animals of the same weight and approximately the same age. The preparations were used to determine the diameters of the axons supplying the flexor muscles of the forearm in both the test animal and the control.

RESULTS. Ten test animals and ten controls were used for the determination of the chronaxie and the rheobase of the flexor muscles of the digits and wrist. The normal variation of the controls was between 0.06 and 0.12 sigma. The rheobase varied between 0.11 and 0.15 volt. The chronaxie of the test muscles varied over the same range as the controls, i.e., 0.06 to 0.12 sigma. The rheobase of the test muscles varied from 0.09 and 0.12 volt—or a slightly lower range than the controls. Table 1 gives the rheobase and the chronaxie of each of five controls and five test animals.

Over one thousand isotonic contractions of the control and test muscles were recorded and measured. The time record was taken from the vibrations of the tuning fork at one hundred per second. These records were plotted graphically, the height of the contraction against the duration in time of the twitch. The duration of the contraction phase of the twitch was measured and compared with the relaxation phase.

The duration of the isotonic twitches varied between 0.11 and 0.13 second. This variation was the same for the control and for the test animals. The duration of the contraction phase of the twitch ranged between 0.07 and 0.08 second. The relaxation phase accordingly varied between 0.04 and 0.05 second. These proportions were also the same for both the control and the test muscles.

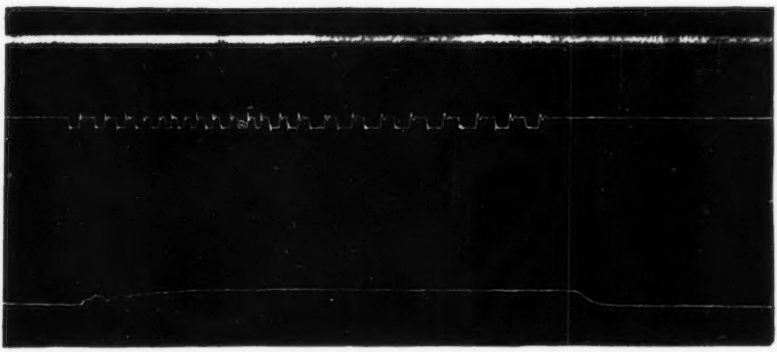


Fig. 1. A kymographic record of a fused tetanus produced by a muscle supplied by regenerated nerves. The course of the lever (lower line) illustrates that only when the stimuli (middle line) are applied to the nerve at intervals of 0.07 second or less (tuning fork vibrating at 100 per sec.) is a fused tetanus produced.

The duration of the isometric twitches of both the control and the test muscles showed less variation than the isotonic contractions. The normal variation was between 0.11 and 0.12 second. The contraction phase of the twitch in both the test muscle and the control was 0.07 second. The tension developed in the contraction twitch was between sixty and seventy grams. In a fused tetanus both test and control muscles developed a tension of about one hundred and twenty-five grams.

The above measurements were further checked by determining the rate at which stimuli had to be applied to the test and control muscles through their nerves to produce a fused tetanus. Stimuli at intervals of 0.07 second caused a fused tetanus. Less frequent stimuli, however, did not result in a complete tetanic contraction of the muscle. Figure 1 is a record taken of a test muscle and further demonstrates this point.

The histological studies of the control and the regenerated nerves support earlier findings. The normal distribution of the axon diameters of the nerves supplying the flexor muscles of the wrist and digits of the forelimb of the albino rat is illustrated in figure 2.

To determine the diameter of the motor axons to these muscles the spinal ganglia were removed from the roots supplying the median and ulnar nerves. Sections of these nerves following the degeneration of the sensory fibers demonstrate that the motor axons to these muscles are between 5.3 and 7.2 micra in diameter. Figure 2 also illustrates the distribution of the diameters of the regenerated axons. These axons, both sensory and motor, are of a strikingly uniform size. They are about 1

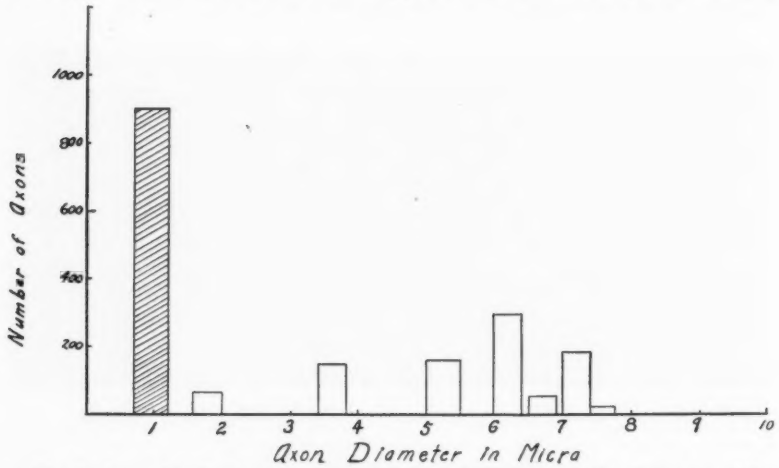


Fig. 2. A graphic illustration contrasting the distribution of the diameters of the axons normally supplying the M. flexor digitorum profundus in the rat with the diameter of the regenerated axons. The plain rectangles represent the normal axons, the cross-hatched rectangle the regenerated axons.

micron in diameter. The size of the sheath appears increased in proportion to the diameter of the axons and the number of axons below the lesions had increased over that found in the control animal. The amount of increase varies with the point at which the section is taken but is about 15 per cent at the point where the axons entered the muscle. No attempt was made to determine the axis sheath relationship.

Whether or not these regenerated axons ever attained a diameter of the normal control has not yet been finally determined. However, twelve months after the animal has regained use of the muscles involved the axons are still about 1 micron in diameter.

Discussion. The results presented here do not support the generaliza-

tions made correlating the time characteristics of a muscle and the diameter of the motor axons which supply it. The physiological characteristics of the muscles examined in these cases are not dependent upon the diameter of their motor axons.

The diameter alone is not an index of the irritability of an axon for the chronaxie of the regenerated axons is the same as that of the normal controls. Judging only from their fiber diameter, however, they would be expected to have a longer chronaxie because of their small size. Lapicque (1929) implied that the irritability of axonal protoplasm may not always be expressed as a function of its diameter.

Biedermann's original observation therefore may apply to sensory nerves as the work of Barnes (1932) would indicate, but certainly there exists no causal relationship between the diameter of the motor axon, the speed of the nervous impulse and the characteristics of the muscle it excites. Muscle irritability aside from the trophic influences of the motor nerves appears to be a property intrinsic to that tissue.

SUMMARY

1. The size of the axons supplying muscles has been changed experimentally by cutting or crushing the nerve and the reactions obtained following functional recovery through nerve regeneration studied.

2. The chronaxie of the flexor muscles of the hand was first determined for both test animals and controls. Both groups fall within the same range with a chronaxie from 0.06 to 0.12 sigma. The rheobase of the test animals was slightly lower (0.09–0.12 volt) than the controls; 0.11 to 0.15 volt.

3. The isotonic contractions of the same group of animals showed the same variation, 0.11 to 0.13 second. The contraction phase (0.07–0.08 second) and the relaxation phase (0.04–0.05 second) were the same for the two groups.

4. The isometric twitch showed less variation in the two groups than did the isotonic contraction, 0.11 to 0.12 second for both series. The contraction phase was 0.07 second, the tension developed was 60 to 70 grams and in a fused tetanus these muscles developed a tension of 125 grams.

5. Histologically the motor axons normally supplying the muscles studied had a diameter of 5.3 to 7.2 micra. The regenerated fibers are approximately all of the same size—about 1 micron.

6. These results do not support the concept of close correlation between the anatomical structure of the motor axons and the physiological characters of the muscles supplied.

7. The diameter of the motor axons supplying a given muscle group may be decreased without affecting the characteristics of the muscle.

8. The facts given above demonstrate the unreliability of axonal diameters as an index of their relative irritability.

BIBLIOGRAPHY

BARNES, T. C. 1932. This Journal, c, 481.
BARRON, D. H. 1932. Thesis. Yale.
BIEDERMANN, W. 1895. Electrophysiologie.
BOURGINGNON, G. 1923. La Chronaxie chez l'Homme. Paris.
CARLSON, A. J. 1905. This Journal, xv, 136.
CASH, J. T. 1880. Arch. f. Anat. u. Physiol., suppl. Bd. 147.
FULTON, J. F. 1926. Muscular contraction and the reflex control of movement. Baltimore.
GASSER, H. S. AND J. ERLANGER. 1926. This Journal, lxxx, 522.
GREENMAN, M. J. 1913. Journ. Comp. Neurol., xxiii, 479.
HAY, J. 1901. Liverpool Med.-Chirurg. Journ., xli, 431.
KREINDLER, A. 1927. Compt. Rend., xcvii, 125.
KRONECKER, H. AND W. STIRLING. 1878. Arch. f. Anat. u. Physiol., Physiol. Abt. 1.
LAPICQUE, L. 1926. L'Excitabilité en Function du Tempts. Paris.
LAPICQUE, L. AND P. DESOILLE. 1927. Compt. Rend., xcvii, 123.
LAPICQUE, L. AND R. LEGENDRE. 1913. Compt. Rend. Acad. Sci., clvii, 1163.
MATHEWS, B. 1929. Journ. Physiol., lxvii, 169.
NICHOLAS, J. S. AND D. H. BARRON. 1932. Journ. Pharm. Exp. Therap., xlvi, 125.
STARLING, E. 1930. Principles of human physiology. Ed. by C. Lovatt Evans. Philadelphia.

ELECTRICAL POTENTIALS IN SMOOTH MUSCLE

A. ROSENBLUETH, C. LEESE AND E. LAMBERT

From the Laboratories of Physiology in the Harvard Medical School

Received for publication October 19, 1932

In recent work by one of us (Rosenblueth, 1932b) the view was adopted that the autonomic nervous impulses liberate a chemical mediator which determines the response of the effector. Previously Cannon and Bacq (1931) had demonstrated the passage into the blood stream of a sympathomimetic substance on stimulation of the sympathetic nerves; they named this substance sympathin. Their experiments, and other subsequent work (see Rosenblueth and Cannon, 1932), showed features of physiological identity between sympathin and adrenin.

In the paper first cited (Rosenblueth, 1932b) it was shown that the muscular responses to repeated nervous stimuli are qualitatively and quantitatively identical with those obtained by different concentrations of adrenin (see Rosenblueth, 1932a).

The chemical interpretation of the transmission of sympathetic nervous impulses clearly separates the processes of excitation and contraction. It was deemed of interest to find out whether the changes of electrical potential in the muscle corroborate this view, by comparing the responses obtained by stimulation of the sympathetic with those evoked by injections of adrenin. Since Cannon and Bacq (loc. cit.) showed that sympathin is still produced after administration of ergotoxine, it was also considered advisable to investigate the influence of this drug on the electric responses.

In reviewing previous work on electrical potentials of smooth muscle several surprising facts were revealed. Funke (1922) gives the literature to that date; more recent papers have been published by Alvarez and Mahoney (1922), Richter (1924), and Hasama (1930 and 1931). Though the number of publications is relatively abundant, the majority of the authors have confined their attention to spontaneous activity and have performed very few experiments on the potentials developed by nervous stimulation.

The authors who have registered both the electrical and the mechanical phenomena agree in stressing the simultaneousness of the two processes (Orbeli and Brücke, 1910; Alvarez and Mahoney, 1922; Funke, 1922; Richter, 1924; and Hasama, 1930). Funke (loc. cit.) states that the electrogram precedes the mechanogram, but his electrical variations last

from 10 to 20 seconds and there is only one for each wave of contraction; he calls them, however, excitation waves. If we consider that it is usually necessary to give several stimuli above a certain minimum frequency to obtain the type of responses with which Funke dealt (see the records of contraction of the stomach and uterus, Rosenblueth, 1932b), it is evident that these single electrical phenomena do not correspond to the repetitive discharges accompanying tetanus of skeletal muscle.

The potentials reported after stimulation of the nerves (Brücke and Oinuma, 1910; Stübel, 1912; and Richter, 1924) show the same discrepancy between the nervous impulses and the electrical phenomena which ensue. The stimuli on the nerves are induction shocks at "tetanizing" frequencies, applied for intervals varying usually between 10 and 30 seconds. No electric response is mentioned for a single shock. The authors describe single or complex potentials developed after many stimuli have been applied, or else discuss the influence of the excitation of the nerves on the previous pattern of spontaneous waves. It is interesting that the smallest unit of time used in all the records mentioned up to now is 1 second, some authors using intervals as long as 12 seconds to measure the curves obtained.

We could find only two instances of potentials recorded after single shocks. Brücke and Inouye (1912) observed that certain leads from the esophagus of the rabbit recorded, after stimulation of the vagus, a complex tracing with two phases, a fast and a slow one. They attributed the first to skeletal muscle, the second to smooth muscle. Unfortunately no mechanograms are given. This, together with the complexity of the preparation employed, makes a judgment impossible. Bozler (1930) registered with an unamplified string galvanometer the potentials developed in the retractor of the pharynx of *Helix pomatia*. The excised muscle was stimulated directly and the mechanograms were taken. The potentials do not precede the contractions, but are simultaneous with them.

It appears, therefore, that electrical potentials comparable with those exhibited by skeletal muscle have not been established for smooth muscle and that the results previously reported are useless for the solution of the problem on hand. A new question then arose: are the electrical potentials exhibited by smooth muscle fundamentally different from those well known in skeletal muscle, or are there similarities which have thus far escaped notice?

GENERAL METHOD. The nictitating membrane, the bladder, the uterus, and the pilomotors of cats were studied. Most experiments were performed under dial anesthesia; a few, however, were done on decerebrate or decorticate preparations after the ether had been eliminated, in order to observe the results in the unanesthetized animal. It was thus found that dial does not modify the responses.

The string galvanometer and the recording camera were those described by Forbes, Davis and Lambert (1930). The amplification was obtained by the two stage condenser coupled unit described by Leese and Einarson (1932). The string was of gilded quartz, its length was 130 mm., its diameter 4μ and its resistance 1,000ω.

The calibration of the amplifier was obtained by applying 50 microvolts to the input with different shunts across the string. The excursions of the shadow of the string in the record were the following:

Shunts in ω	*Excursions in the record in mm.*
100	4.5
200	8.0
300	11.0
400	14.0
500	16.5

The stimulating device consisted of a Harvard inductorium with a 1.5 volt dry cell in the primary circuit. Makes and breaks were produced by means of a spring key with a mercury contact. Shielded stimulating electrodes were applied to the nerves cut or crushed centrally.

The relative effectiveness of the make and break shocks as stimuli varied with the preparations, the break being stronger but briefer. As a rule the make was found to be more suitable. A key was inserted in the secondary circuit which could short-circuit either shock. In all cases the intensities employed were the minimum which would give a definite response.

The leads to the galvanometer were either sharpened silver wires with chlorided tips or, likewise chlorided, fine concentric needle electrodes similar to those described by Adrian and Bronk (1929). The concentric electrodes were arranged so that the exterior served as the ground connection, while the insulated core was connected to the grid lead. The first type of leads mentioned permits the recording of potential changes in a relatively large portion of the muscle, whereas the second type is affected by only a few cells.

We generally avoided shielding to reduce the artefacts produced by the electrical stimulus, as they served to show the moment the nerve was stimulated. The size of the artefacts varied with the intensity, the amplification and the preparation. The shape was, however, always comparable. Several controls were taken by recording artefacts in the dead animal. Figure 1 illustrates the common types of artefacts obtained.

A light torsion myograph was employed, which threw a shadow on the film. Since we were interested only in the time relations between the electrogram and the mechanogram, no special precautions were taken as to the type of contraction which resulted. For the nictitating membrane

the myograph was set up so that a tension of 1 gram produced an excursion of 11 mm.; the corresponding shortening of the muscle was 3 mm. For the uterus, the adjustment was such that a tension of 1 gram produced an excursion of 7.5 mm. in the record and a shortening of 2 mm. in the muscle. For the bladder, the same tension produced an excursion and a shortening between the previous figures. Within limits respected in the experiments, excursions in the record and shortenings of the muscles were lineally proportional to the tension developed. The initial tensions applied to the muscles were the following: on the membrane, 1 to 1.5 grams; on the bladder, 2 to 3 grams; on the uterus, 3 to 6 grams. The movement being always slow and the inertia of the myograph small, the time relations are reliable. In the uterus, the leads to the galvanometer were usually placed in the vicinity of the hook attached to the myograph. Since, however, the responses of the pregnant viscus have a tendency to travel as waves and

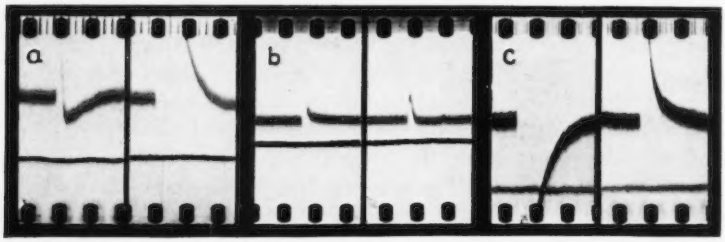


Fig. 1. Artefacts. Time: 10σ. In each case a make and a break are included.
A. Concentric leads on the nictitating membrane, 500ω shunt.
B. Diffuse leads on nictitating membrane, 100ω shunt.
C. Diffuse leads on uterus, 100ω shunt.

since the structure of the muscle is complex, the correlation between the electrogram and the mechanogram is not very strict.

The solutions of cocaine, adrenalin, and ergotoxine were injected intravenously.

RESULTS. I. ELECTRICAL POTENTIALS DEVELOPED BY STIMULATION OF THE SYMPATHETIC. A. *Nictitating membrane.* Stimulating electrodes were applied to the cervical sympathetic at about the level of the thyroid cartilage. We were thus stimulating preganglionic fibers to the nictitating membrane, and the approximate length of nerve the impulses had to travel was about 7 cm. The average total latency before the beginning of contraction was usually about 410σ.

Two distinct electrical disturbances appear consistently in our records (fig. 2). The first one, which we shall call the initial potential, invariably precedes the contraction. Its latency is usually about 90σ; its shape in the records varies with the leads and the place where they are inserted;

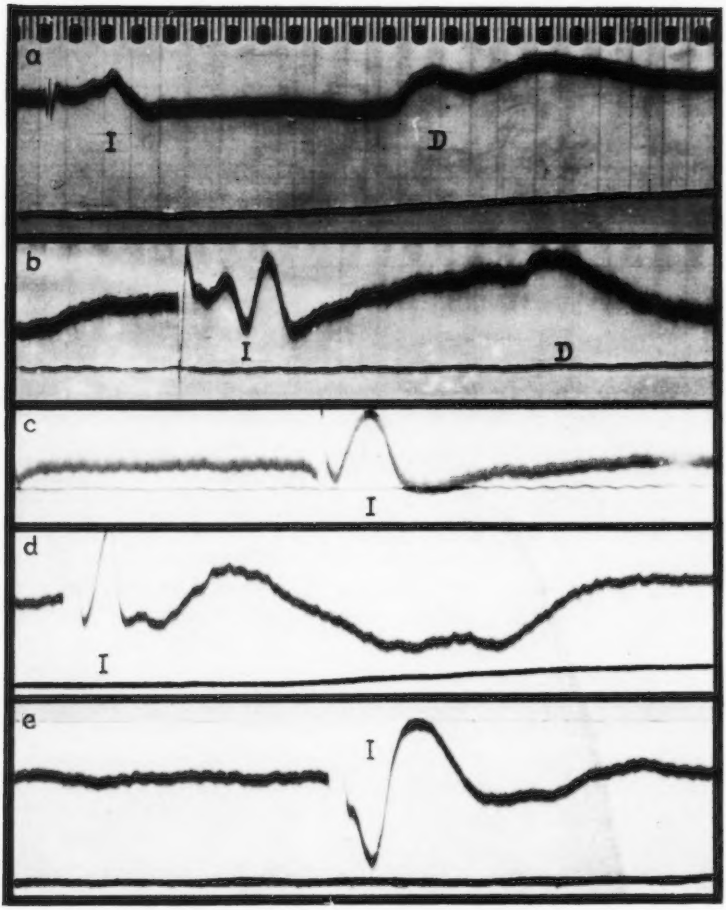


Fig. 2. Different types of initial and delayed potentials from the nictitating membrane. Time in all records: 10σ. In this and other figures initial potentials are marked *I*; delayed potentials are marked *D*; the narrower tracing is the shadow of the myograph.

a. Concentric leads, 100ω shunt.

b. Concentric leads, 300ω shunt. This record was taken from the middle of a series; the membrane was half contracted.

c. Diffuse leads, grid on muscle; 100ω shunt.

d. Diffuse leads, grid on muscle; 100ω shunt.

e. Same leads and shunt as in *d*, but ground on muscle.

but, for a given preparation and recording set-up, both latency and shape are quite consistent, both on the makes and on the breaks. Quite pure monophasic records were sometimes obtained (fig. 2-c and d), whereas in other cases the record presents a polyphasic complex (fig. 2-a and b).

The second or "delayed" potential occurs during contraction. It is more variable than the initial potential, both in latency and shape (fig. 2), but, like the initial, it is quite consistent in a given preparation. The two potentials are perfectly distinct in some records (fig. 2-a); in other cases they appear to form a continuous curve (fig. 2-e). The delayed potential usually begins simultaneously with contraction in the mechanogram. In some cases, however, it seems to precede the rise slightly; it is difficult to decide whether this is actually the case, for it is hard to determine the exact moment at which contraction begins. At other times the delayed potential is recorded definitely after the beginning of contraction. This description of the delayed potentials applies only to those evoked by a single shock producing a twitch of the muscle. During a series of stimuli at an adequate frequency—2 or 3 shocks per second, for instance—the delayed potentials do not appear separately for each shock, but fuse throughout the tetanus, so that the duration of the potential change becomes very long. We did not run the camera slowly enough to obtain proper records of this type of electric response.

It is interesting that a certain set-up of the recording system may give exclusively either initial or delayed potentials. Frequently the typical electrogram is evoked only by some of the stimuli, the others giving nothing but pictures of artefacts; it is then invariably seen that contraction occurs only after those stimuli which evoke electric responses.

When series of stimuli are applied at a frequency sufficient to produce a summation of the mechanical response, the artefacts will, of course, not show any variations; the electric responses, on the contrary, show a progressive, irregular decrease in size and may, in some instances, disappear altogether.

B. *The pilomotors.* The electric responses were recorded from some point in the proximal third of the tail. The recording leads were usually the concentric electrodes introduced superficially. The stimulating electrodes were applied to the abdominal sympathetic chains at about the level of L5, giving an approximate length of nerve of 18 cm. No mechanograms were obtained but the latency for the erection of the hairs can be roughly estimated by sight as about half a second.

Beautiful initial potentials (fig. 3-a) are readily obtained. Their latency is approximately 180σ, very constant for a given preparation. The distinction between artefact and response is complete, because of the long latency.

The delayed potentials are more difficult to obtain. They are shown in

figure 3-b. The remarks made about the two types of potential changes in the nictitating membrane are applicable also to the pilomotor responses.

C. *The bladder.* The stimulating electrodes were applied to the hypogastric nerves immediately below the inferior mesenteric ganglion. This gives a nerve length of about 6 cm. for the impulses to travel.

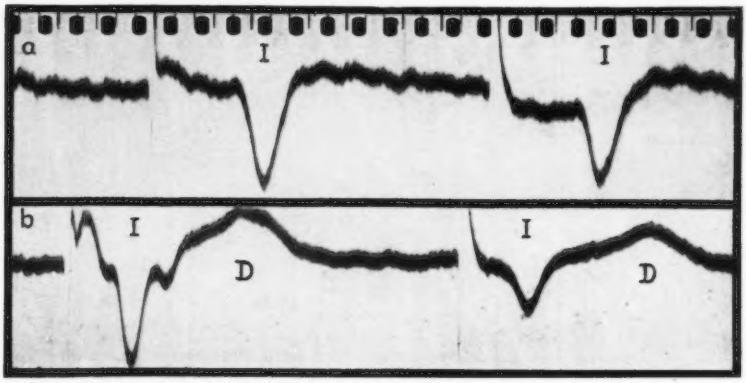


Fig. 3. Initial and delayed potentials from the pilomotors. Time: 40σ.

a. Responses to a make and a break taken from the middle of a series. Concentric leads, 200ω shunt.

b. Same as *a* in another preparation, 100ω shunt. See discussion for probable explanation of the complex initial potential on the break.

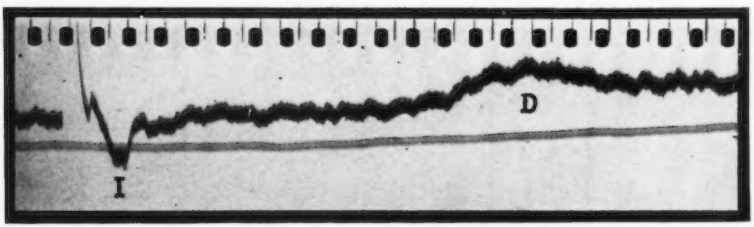


Fig. 4. Initial and delayed potentials from the bladder on a make shock. Concentric leads, 200ω shunt. Time: 40σ.

Here both electric responses are difficult to record, especially the initial potential. We found the concentric electrodes much more suitable for the purpose than the ordinary, diffuse leads. Figure 4 illustrates the records obtained. The latency for the initial potentials was usually around 60σ. The myograph was attached to the free vertex of the empty bladder and readily recorded contraction, since it is mainly the trigonum and the detrusor which respond. As Stewart (1900) showed, a single

induction shock will evoke a neat twitch. The latency for the muscular response was usually about 360σ in our observations. Here again the behavior of both types of electric responses is comparable to that found in the nictitating membrane or the pilomotors.

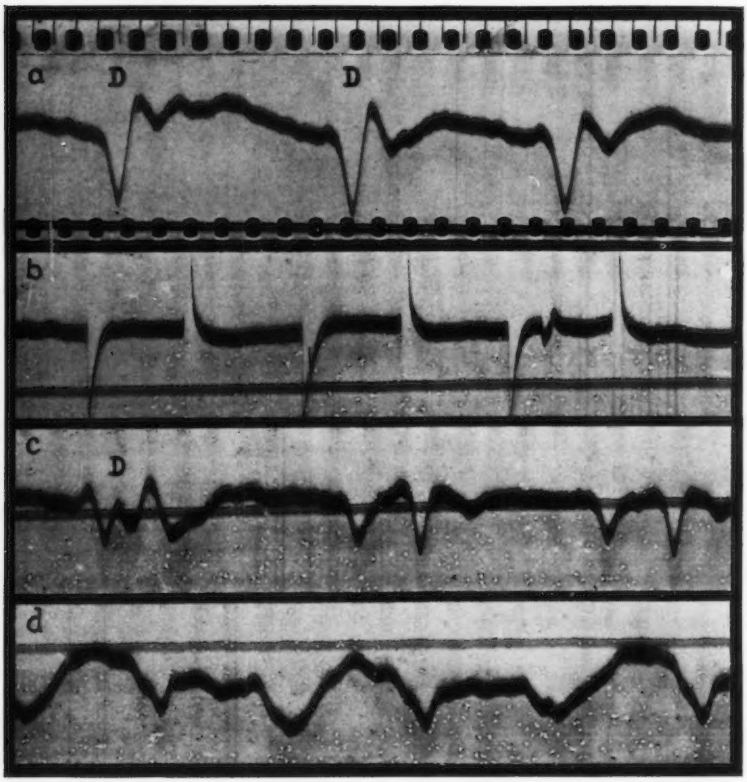


Fig. 5. Delayed potentials from the uterus. Time: 40σ.

a. During a spontaneous contraction. Diffuse leads, grid on muscle, 100ω shunt.

b. Absence of initial potentials during repeated stimulations. The end of a series lasting 10 seconds. Diffuse, leads, 100ω shunt.

c. Fifteen seconds after *b*.

d. Ten seconds after *c*, at the peak of the contraction.

D. *The pregnant uterus.* The stimulating electrodes were applied to the hypogastrics as in the case of the bladder. The probable length of nerve involved was about 8 cm. to the regions where the recording leads were usually inserted, near the junction of the two horns. The results

are entirely different from those obtained on the other structures. A single shock never elicited any response whatsoever (fig. 5-b). We were not able to trace even an approximation to an initial potential comparable to those previously described. Much greater amplifications were tried than those used in the other organs, but without success.

A series of stimuli (2–15 per second) does develop, however, an electric response. The latency depends on the frequency of the impulses, being shorter with briefer intervals between the stimuli. The electrogram then

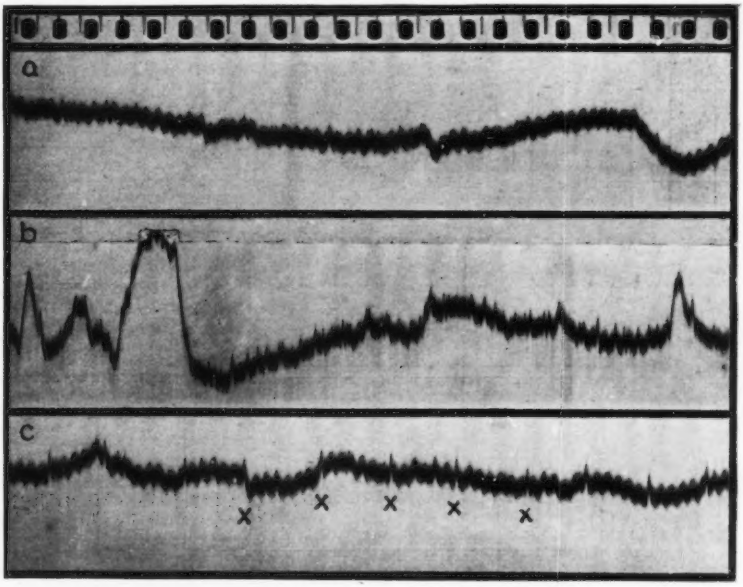


Fig. 6. Initial potentials from the pilomotors in the tail during asphyxia. Decerebrate animal out of ether. Concentric leads, 200ω shunt. Time: 40σ.

a. Base line before asphyxia.

b. Beginning of the erection of the hairs.

c. Full erection. The crosses mark the initial potentials.

presents a series of slow, usually regular complexes (fig. 5-c and d). These responses are similar to those we obtained during spontaneous contraction (fig. 5-a) and to those previously described by other authors. The electric responses being simultaneous with the contraction (fig. 5-c and d), we shall class them as delayed potentials. The significance of these phenomena will be taken up in the discussion.

II. Asphyxia and reflex or spontaneous sympathetic discharges in the decorticate animal. These preparations were used to avoid

electric stimulation and to bring forth nervous discharges of central origin, therefore presumably out of phase with one another (see Adrian, Bronk and Phillips, 1932). Figures 6, 7 and 8 show the results obtained. The initial potentials are evident. Figure 8-b shows a rate of 17 nervous impulses per second. This rate is adequate to bring forth a maximal response (see Rosenblueth, 1932b). The recording outfit was adjusted mainly for the demonstration of these initial potentials. This requires a relatively high speed in the camera, unsuitable to show slow delayed potentials (see p. 664 and discussion).

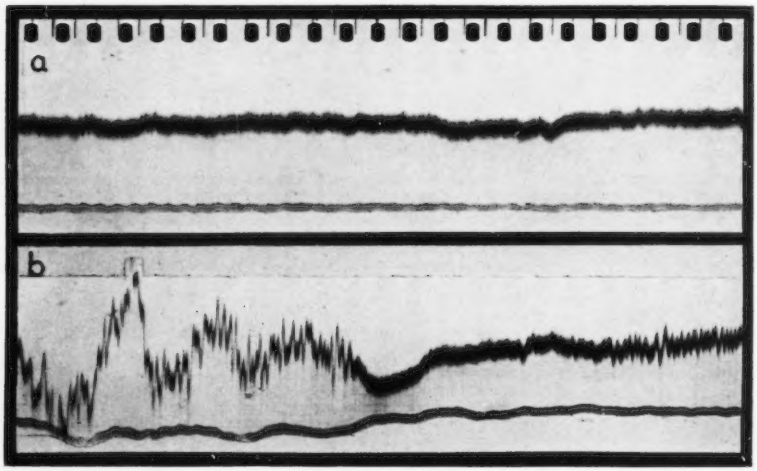


Fig. 7. Initial potentials from the nictitating membrane during asphyxia. Decerebrate animal out of ether. Diffuse leads, 200ω shunt. Time: 40σ.

a. Base line before asphyxia.

b. During the contraction of the membrane. The irregularities in the mechanogram are due to movement of the animal.

III. THE INFLUENCE OF COCAINE. Since cocaine was used in several experiments to increase the responses of the nictitating membrane to adrenalin, a few controls were taken by recording the responses to nervous stimulation before and after the drug. No differences were observed in the electrical effects (figs. 10-a and 11-a). The mechanical response, on the other hand, is considerably increased (see Rosenblueth and Rioch, 1933).

IV. THE ACTION OF ADRENIN. Records were taken from the nictitating membrane when contracting as a consequence of injected adrenalin. Some observations were made on membranes denervated about ten days

previously by removal of the superior cervical sympathetic ganglion. Other experiments dealt with the same preparations used while stimulating the cervical sympathetic; in this case cocaine was usully injected to insure larger responses to adrenin. The latter series of observations could be

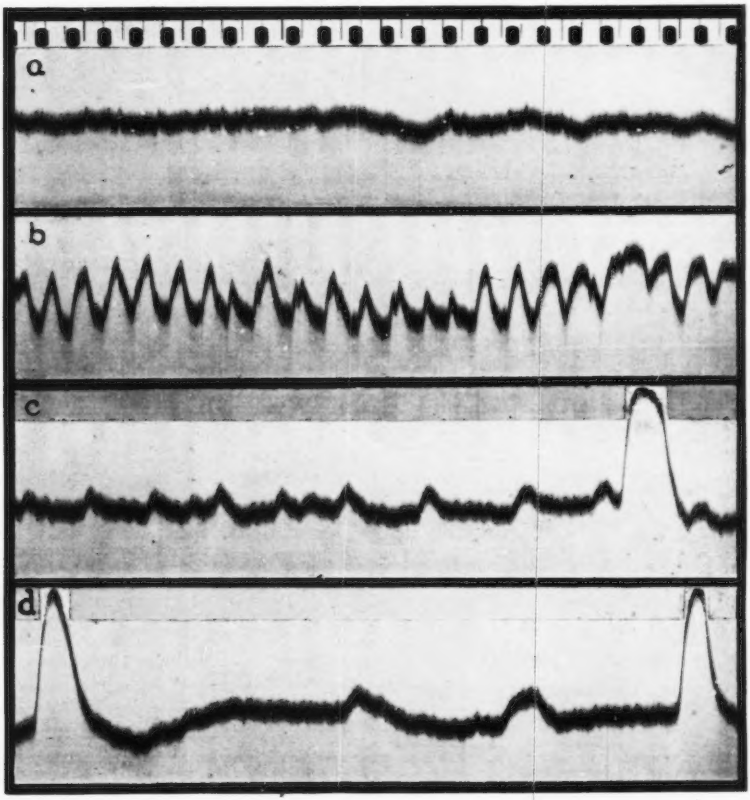


Fig. 8. Initial potentials from the pilomotors in the posterior upper thoracic region of the animal during the reflex contraction brought about by pinching a decorticate preparation. Concentric leads, 200ω shunt. Time: 40σ.

a. Base line. Hairs flat.

b. Two seconds after mechanical stimulus. Hairs erecting.

c. Three seconds later. Hairs fully erected.

d. Five seconds later.

controlled as to the proper position of the recording leads by photographing potentials induced by nervous stimulation before and after the injections of adrenalin.

The records are not simple to interpret because of several causes of error which will be dealt with in the discussion. In no case, however, either

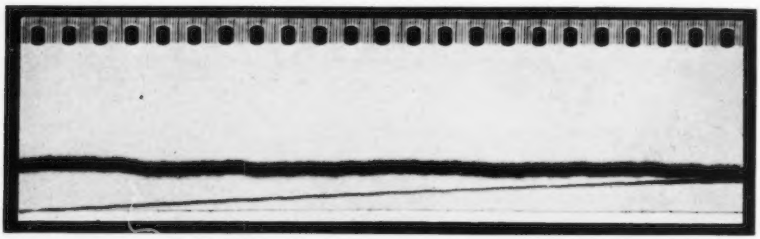


Fig. 9. Absence of initial potentials in a denervated nictitating membrane contracting under the influence of adrenalin. Diffuse leads, 500ω shunt. Time: 10σ.

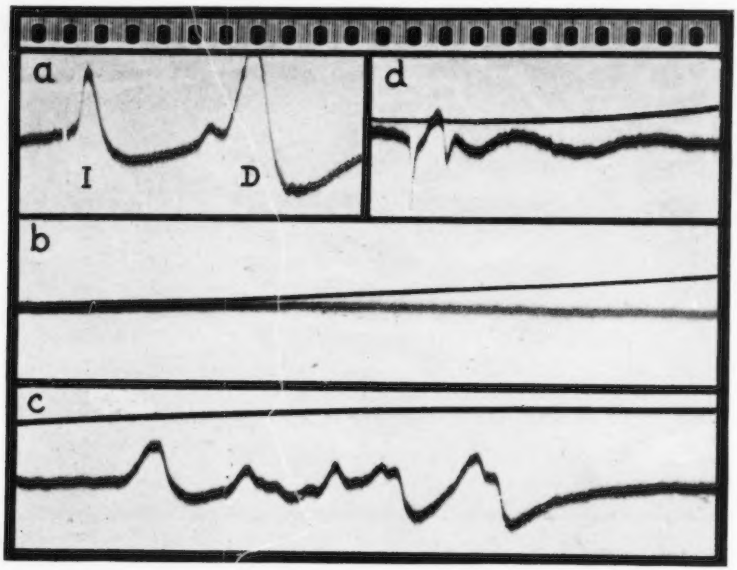


Fig. 10. Absence of initial potentials during action of adrenalin on the nictitating membrane. Cocaine. Time: 10σ. 500ω shunt.

a. Initial and delayed potentials obtained by stimulating the nerves before administration of adrenalin.

b. Beginning of contraction with adrenalin.

c. At the height of contraction. These irregular excursions were isolated, the rest of the record being smooth.

d. Initial potentials on stimulation of nerves. Membrane not completely relaxed after the adrenalin.

with diffuse or with localizing leads, were any definite initial potentials obtained. We reproduce in figure 9 a typical smooth tracing from a denervated membrane. Figure 10-c shows a few irregular excursions which appeared in an otherwise smooth record and are probably due to causes other than functional activity. There never was any indication of repetitive activity comparable to that shown in figures 6, 7 and 8. This difference between adrenalin and asphyxia is further emphasized by figure 11.

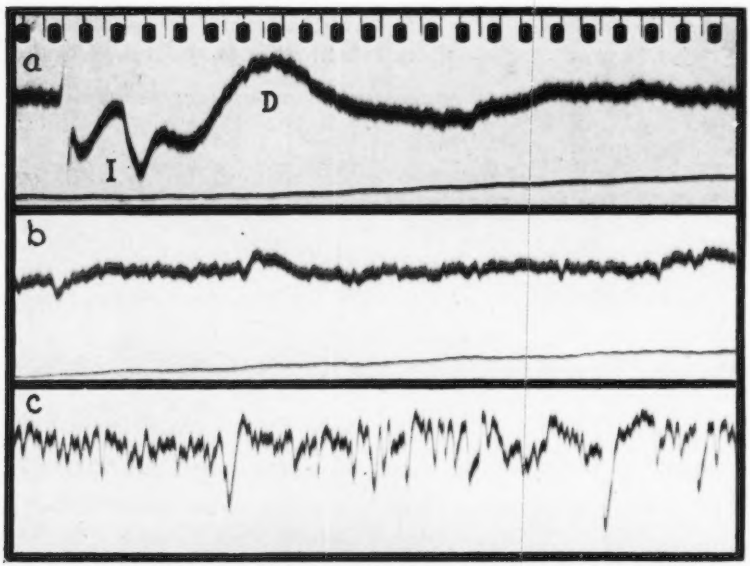


Fig. 11. Absence of initial potentials in the nictitating membrane when contracting with adrenalin and existence of these potentials when contracting during asphyxia. Concentric leads, 400ω shunt. Cocaine. Time: 40σ.

a. Electrical stimulation of the cervical sympathetic.

b. Beginning of rise with adrenalin.

c. Beginning of rise with asphyxia. The myograph was not attached.

The effect of adrenin on the uterus is shown in figure 12. The electric disturbances appear during contraction and are closely similar to those presented by spontaneous activity (fig. 5-a) and after repeated nervous stimulation (fig. 5-c and d).

The effect of adrenin on the pilomotors was investigated in two ways. As is well known, the contraction of the pilomotors is difficult to obtain in most animals by intravenous injections of adrenin. Subcutaneous injection in the tail of the cat, however, gives a definite response in a small

area surrounding the injection. The observation had also been made by one of us (Rosenblueth) that intravenous injection of a rather large dose (about 0.1 mgm. per k.) of adrenalin will evoke a maximal, long-lasting erection of the hairs of the tail of the woodchuck (*Marmota monax*).

Both methods were tried with negative results, i.e., no deflections appeared on the electrogram. Stimulation of the abdominal sympathetic in the woodchuck gave electric responses of the pilomotors analogous to those obtained in the cat.

V. THE ACTION OF ERGOTOXINE. The effects of this drug are complex and vary in different organs. On the nictitating membrane, paralysis of the responses to nervous stimuli is obtained with doses of about 0.25 mgm.

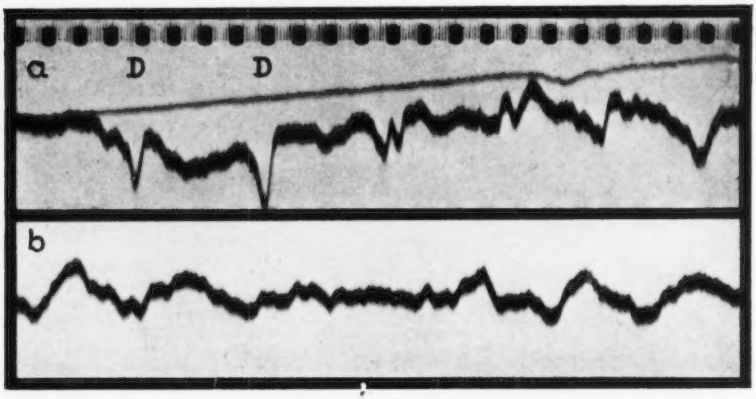


Fig. 12. Delayed potentials from the pregnant uterus during contraction produced by 0.5 cc. adrenalin 1:50,000. Diffuse leads, grid on uterus. 500ω shunt. Time: 10σ.

a. Beginning of contraction.

b. Four seconds after *a*. The shadow of the myograph was beyond the top of the film.

per k. The membrane develops slight contracture (Gasser, 1930). Further doses increase the contracture. On the pilomotors we found that with doses of about 0.5 mgm. per k. the responses to nervous stimuli are at first rapidly decreased. Whereas a visible erection occurs with a single shock before the injection, a series of about 20 stimuli at a rate of 2 per second is necessary afterwards to elicit any contraction. If the doses are increased, longer and more frequent successions of stimuli are necessary to evoke the slight mechanical response. We were not able, however, to paralyze completely the erection; neither did any appreciable contracture show itself.

The base line of the galvanometer usually became rougher after ergo-

toxine. The initial potentials from the nictitating membrane generally disappeared as soon as contracture and complete paralysis were obtained. Frequently, with small doses which did not paralyze the contraction completely, a good many stimuli would evoke no response, and then an apparently normal initial potential would show itself. With larger doses and consequent contracture and full paralysis some preparations exhibited complex records; the tracings are, however, so different from the ones commonly obtained that a verdict could only be tentative.

The action of ergotoxine on the electric responses of the pilomotors is simpler. The excitation potentials persist to the end. A slightly greater amplification is necessary to make them comparable to those obtained before administration of the drug. The deformation of the records is slight and may be accounted for by progressive injury of the tissues in contact with the leads and by shifts of position due to slight movements of the electrode. Figure 13 illustrates an example.

Discussion. The significance of the electric responses of muscle is still a matter of debate. In the exposition of our results we have adopted the dual interpretation, in which the electromyogram is made of two parts, one corresponding to excitation, the other to contraction. This view was first formulated by Schenck (1895) and has been recently defended by Bishop and Gilson (1927) who employ the terms "excitation and action potentials." We shall immediately state the reasons which led us to this attitude (see Gasser, 1930, and Lindhard, 1931, for other interpretations of the electromyogram).

We have shown in the introduction that the potentials recorded from smooth muscle by previous workers are not analogous to those obtained from skeletal muscle because the former coincide with the mechanical response and because, when the nerves were stimulated, there is not a separate electric response for each nervous impulse. Our records on the uterus (fig. 5) are closely similar to those found by other investigators.

With the exception of the retractor penis, the preparations used previously were, however, complex and therefore not well suited to reveal the phenomena occurring. We found it difficult to lead the electric responses from the bladder (see p. 665) and the records obtained (fig. 4) are not as neat as those of the nictitating membrane (fig. 2) or the pilomotors (fig. 3). These last two organs are ideal in their simplicity, having parallel muscle cells and showing a definite response to a single stimulus.

The initial potential in the membrane and the pilomotors is strictly comparable to the spike in the "action current" of skeletal muscle. The phenomenon precedes contraction. Einthoven (1924) pointed out a source of error for the determination of the time relations in records obtained from skeletal and cardiac muscle: the myograph has usually a greater slackness than the galvanometer. These muscles develop ten-

sion rapidly and this error may then be considerable. In smooth muscle, however, where contraction is relatively very slow, this error may be disregarded.

The possibility of our dealing with potentials of nervous origin was considered and dismissed for the following reasons. The number of nerve fibers from which the leads, especially the localizing Adrian and Bronk concentric electrodes, could transmit potentials is too small; in the pilomotors these fibers would probably be only one or two. The magnifications used (see p. 661, the calibration of the amplifier) were not sensitive enough to record the electric responses of a few nerve fibers (Adrian, 1926). Adrian, Bronk and Phillips (1932) have given reasons to believe that the potentials developed by sympathetic fibers are smaller than those obtained from somatic nerves. In one instance the concentric electrodes were placed in the superior cervical ganglion; the potentials recorded were similar in size to those obtained from the membrane or the hairs; but in this case a much greater number of nervous elements were probably in closer contact with the electrodes than in the muscles. The potentials recorded from the muscles are therefore too large to be of nervous origin.

If the initial potentials were of nervous origin considerable difference would appear between a volley and a discharge out of phase; indeed, in the latter case, the phenomenon would probably disappear. Figure 8-b shows the persistence of the electrical potentials in reflex discharges, which presumably occur out of phase.

Our failure to obtain initial potentials from the uterus is another argument against their nervous origin. Out of about 100 different observations at least a few must have been in a position to record potentials from the nerves, yet the galvanometer did not reveal them.

In one case we believe we were concerned with a potential of spurious origin. In figure 3-b the break shock was spreading to neighboring structures. The earlier beginning of the electric response following the break shocks as compared with the makes was probably due to involvement of neighboring skeletal muscle.

The delayed potentials from the nictitating membrane and the pilomotors appear too consistently to be extraneous or accidental. Mechanical shift of the leads would not give identical pictures throughout a long series of stimuli. This possible source of error was further avoided in some instances by increasing the tension of the myograph, so that the shortening of the muscle was considerably reduced; the delayed potential persisted.

The results in complex muscles (uterus, stomach, etc.) now become clear. What other investigators had previously reported, and what we found in the uterus (fig. 5) are delayed potentials. They appear as rhythmic complexes because the mechanical responses of these muscles are likewise

rhythmic. Though the myograph records in these experiments show a relatively smooth rise, the uniform shortening is the resultant of a series of travelling waves. That this is true is shown more clearly in more accurate myograms (see Rosenblueth, 1932a, b). This type of response contrasts with the smooth tetanic curve obtained from the nictitating membrane (see same papers). In this case (see p. 664) the individual delayed potentials for each shock fuse and a long single deflection occurs, which is not reproduced in our records, as the shadow of the string then frequently passes off the films. On the other hand, a very slow potential change of this sort may leak through the ground connection of the string without causing marked excursions in the film.

Our inability to obtain initial potentials from the uterus (see p. 667 and fig. 5-b) may be explained in two ways. Either this response does not exist in uterine muscle or it is so small that it may not be revealed by the apparatus employed. We incline to think the latter explanation more probable, else it would be necessary to assume that the nervous excitation of this and perhaps other muscles is entirely different from that of the other systems studied in this paper, which is unlikely. The fact that no apparent contraction ensues from a single shock on the nerve may be related to this absence of the initial potentials (see Rosenblueth, 1932b, for a discussion of the "iterative systems" of Lapicque and Meyerson, 1912).

If the existence of two different potential changes in the electric responses of muscle is not generally recognized, it is probably due to the greater difficulty in analyzing the more rapid changes in skeletal muscle, which has been mainly studied. Smooth muscle gives us, so to speak, a slow motion picture of the processes involved, simpler to interpret.

Previous authors have discussed extensively the shape of the potentials obtained from smooth muscle and whether the curves were mono- or polyphasic, and have drawn several conclusions therefrom. Recent work on skeletal muscle has shown that these characters depend on the position of the leads, the amount and state of the tissue in contact with the leads, and the simplicity or complexity of the muscle, and that actually the monophasic response is almost impossible to obtain (see, for instance, Samojloff, 1925; Adrian, 1925; Bishop and Gilson, 1927). We shall therefore not deal with this aspect of the question further.

If the two potential changes (initial and delayed) are admitted and if one of them corresponds to the process of excitation, and the other to that of contraction, the possibility of separating the electrical phenomena, i.e., of obtaining the one without the other, depends on the possibility of separating the two processes. The term "excitation" is here used in a very general sense; it refers to everything occurring between the arrival of the nervous impulse at the muscle and the beginning of contraction; it in-

cludes, therefore, the phenomenon of conduction in the muscle, with which we are not directly concerned in these experiments.

The separability of the two phenomena has been extensively discussed (see Fulton, 1926, and Gasser, 1930, for reviews on the subject). Both Gasser and Fulton conclude that the evidence presented seems to show that conduction cannot take place without contraction. Gasser adds that, "on the other hand, every contracture is an instance of contraction without conduction." Bishop and Gilson (1927) state: "it might be expected that whatever prevented the irritable mechanism from functioning should prevent the subsequent contraction, but it does not follow that factors which depress the contractile function necessarily destroy irritability for conduction of any impulse." The last statement has been shown to be true for the heart by Bogue and Mendez (1930).

As mentioned in the introduction, the chemical theory of the mediation of autonomic nervous impulses developed by one of us (Rosenblueth, 1932b) implies the possibility of separation of the two processes, as follows. The excitatory stage ends with the production of the mediator, the contractile phase begins at once after this mediator diffuses in the cell. The initial potential precedes or is simultaneous with the production of the mediator. The delayed potential attends contraction. It seems plausible that if the mediator is supplied to the cells without stimulating their nerves the initial potential should vanish, contraction occurring, however, normally. If, on the other hand, the contractile mechanism is impaired, or the mediator is prevented from diffusing in the cell, the initial potential should persist, without eliciting any contraction. The first situation is evidently realized by injections of adrenin. As regards the second possibility, the experiments of Cannon and Bacq with ergotoxine, mentioned in the introduction (see p. 659), strongly suggest the probability of obtaining initial potentials without ensuing contraction. We cannot be conclusive in interpreting the results of either set of experiments. The sources of error are numerous and we were not able to control entirely some of them.

In the records obtained with adrenin, there appear no initial potentials (figs. 9, 10 and 11). Several explanations suggest themselves for this absence. It is conceivable that the potentials were too small for our set-up to record (see p. 675). This explanation is, however, not probable, for, as Adrian showed (1922), the electric responses in skeletal muscle obey the all-or-none law. It is possible that initial potentials of muscular origin do not appear with adrenin because they do not exist in smooth muscle. The initial potentials recorded after nervous stimulation would then be of nervous origin and adrenin does not stimulate the nerves. The reasons for rejecting the nervous origin of the potentials have already been stated (see p. 674). This hypothesis seems, therefore, improbable. It is finally possible that the stimulation by adrenin causes the elements to discharge

out of phase with one another so that the electrical effects cancel each other, whereas nervous stimulation gives rise to a volley in which the electrical effects are additive. This is, in our minds, the strongest argument which can be raised to invalidate the interpretation of the results obtained.

The following facts, however, oppose the last view. The concentric leads localize considerably the region investigated; the mutual cancellation

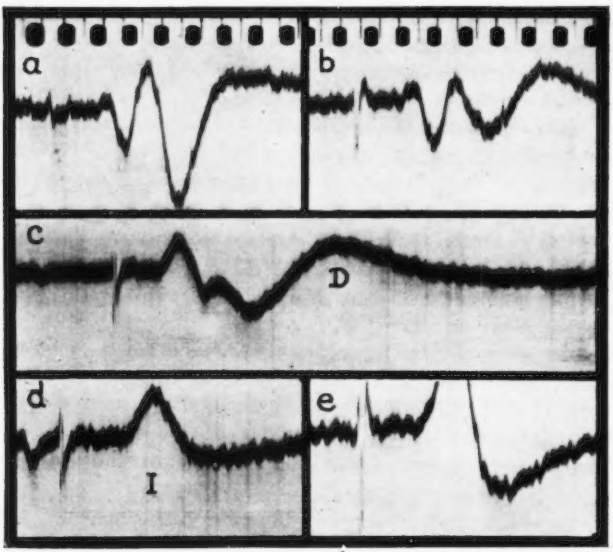


Fig. 13. Persistence of initial potentials in the pilomotors after relative paralysis is obtained with ergotoxine. The contraction nearly disappeared after the first dose. Concentric leads. Time: 40σ. Weight of the cat, 3k.

a. Before ergotoxine, 50ω shunt.
b. After 1.5 mgm. ergotoxine, 100ω shunt.
c. After 3.5 mgm. ergotoxine, 100ω shunt.
d. After 8.5 mgm. ergotoxine, 100ω shunt.
e. After 8.5 mgm. ergotoxine, 200ω shunt.

of the electric responses is therefore considerably lessened. These leads do not reveal the initial potentials after adrenin (fig. 11). In asphyxia and in the decorticate preparation the discharges, being of central origin, do not occur probably in volleys, but out of phase. The initial potentials are, however, perfectly apparent (figs. 6, 7, 8 and 11).

It could, however, be argued that the nervous impulses of central origin could produce simultaneous excitation of larger groups of cells, because of

the connections of one preganglionic fiber with many postganglionic elements. The responses of the muscle cells to adrenin might then be more out of phase with one another than when subjected to nervous impulses. This is one of the difficulties which we did not control. Its importance, however, seems to us secondary.

This discussion of the action of adrenin assumes that the hormone is a physiological stimulant of the muscle, which can hardly be doubted, and that the response elicited is a contraction quite comparable to that obtained through nervous impulses. For further comments on this subject see Rosenblueth and Rioch (1933).

The delayed potentials obtained by adrenin are strictly similar to those occurring after a series of stimuli on any of the structures studied (see p. 671). We therefore believe that when adrenin acts on smooth muscle initial potentials do not exist.

After ergotoxine, the behavior of the nictitating membrane and of the pilomotors differs. The membrane is paralyzed to the nervous stimuli, and the initial potentials tend to disappear (see p. 672). This can be explained because of the contracture of the membrane, which does not exist in the pilomotors. A muscle in contracture is absolutely or relatively refractory to the nervous impulses (see Gasser, 1930). We may dismiss, therefore, further consideration of the membrane. The hairs are not entirely paralyzed, although the mechanical response is almost abolished; nevertheless, the initial potential persists throughout and is practically undiminished (fig. 13). Here, then, there is an almost complete separation of the initial potential and the mechanical response. Some other structure or some other drug may be found, which will show a complete independence of the two processes.

The electrical corollaries of the theory of chemical mediation of nervous impulses are then largely realized. On the other hand, none of the evidence presented is contradictory to the theory.

SUMMARY AND CONCLUSIONS

The electric responses of the nictitating membrane, the pilomotors, the bladder and the pregnant uterus of the cat were studied. The muscles were stimulated through their sympathetic supply or by means of injected adrenin. The influence of ergotoxine on the potentials developed was likewise investigated.

It is shown that the electrical disturbances attending contraction of smooth muscle which have been previously reported are not comparable to those exhibited by skeletal muscle.

Two distinct electrical phenomena of muscular origin were observed in these experiments after stimulation of the sympathetic: an initial potential, which precedes contraction, and a delayed potential, simultaneous

with the mechanical response. The initial potential is most readily recorded from the pilomotors (fig. 3); in the nictitating membrane it occurs quite constantly (fig. 2); it is harder to obtain from the bladder (fig. 4); we were finally unable to find it in the uterus (fig. 5). As regards the facility for obtaining the delayed potential the order of the muscles is the following: uterus, nictitating membrane, bladder, pilomotors (see the same figures).

The initial potential is the equivalent of the spike in the action potential from skeletal muscle. The delayed potential is comparable with the second part of the electromyogram found by Bishop and Gilson (1927) with the Braun tube oscillator and with the T wave of the electrocardiogram. The potentials previously recorded from smooth muscle by other investigators are delayed potentials only. It is pointed out that a simple preparation of smooth muscle, e.g., the nictitating membrane or the pilomotors, is more favorable than skeletal or cardiac muscle to study the different elements of the electromyogram and their time relations with the mechanogram, because the responses are slower and longer.

The source of the electric responses obtained is discussed. A nervous origin is shown to be improbable (see p. 674). The potential changes recorded occur then in the muscles.

Adrenin was found to give rise to delayed potentials only, without initial spikes revealing a conducted disturbance previous to contraction (figs. 9, 10, 11 and 12). Possible explanations for the absence of initial potentials are discussed on pages 676, 677, 678. It was considered that the most serious source of error in this case was the possibility of initial potentials occurring out of phase and thus cancelling one another. To control this possibility asynchronous nervous discharges during asphyxia and reflex activity of the sympathetic were tested out. The typical initial potentials, however, appeared readily (figs. 6, 7, 8 and 11).

Ergotoxine was found to leave practically intact the initial potentials on nervous stimulation of the pilomotors, while impairing considerably the mechanical response (fig. 13 and pp. 672, 678).

It is concluded that the initial potential is simultaneous with the production of sympathin while the delayed potential is a function of contraction. It is further concluded that the initial potential and contraction are separable phenomena. Adrenin and ergotoxine effect this separation.

It is a pleasure for us to thank Dr. Alexander Forbes for his valuable advice and criticism in relation to this work.

BIBLIOGRAPHY

ADRIAN, E. D. 1922. Arch. Neer. de Physiol., vii, 330.
1925. Journ. Physiol., lx, 301.
1926. Ibid., lxi, 49.
ADRIAN, E. D. AND D. W. BRONK. 1929. Ibid., lxvii, 119.
ADRIAN, E. D., D. W. BRONK AND G. PHILLIPS. 1932. Ibid., lxxiv, 115.
ALVAREZ, W. C. AND L. J. MAHONEY. 1922. This Journal, lviii, 476.
BISHOP, G. H. AND A. S. GILSON. 1927. Ibid., lxxxii, 478.
BOGUE, J. Y. AND R. MENDEZ. 1930. Journ. Physiol., lxix, 316.
BOZLER, E. 1930. Zeitschr. f. Vergl. Physiol., xii, 579.
BRÜCKE, E. T. v. AND T. INOUYE. 1912. Pflüger's Arch., cxlv, 152.
BRÜCKE, E. T. v. AND S. OINUMA. 1910. Ibid., cxxxvi, 502.
CANNON, W. B. AND Z. M. BACQ. 1931. This Journal, xcvi, 392.
EINTHOVEN, W. 1924. Harvey Lecture, xx, 111.
FORBES, A., H. DAVIS AND E. LAMBERT. 1930. This Journal, xcv, 142.
FULTON, J. F. 1926. Muscular contraction. Baltimore.
FUNKE, H. H. 1922. Arch. Neer. de Physiol., vi, 198.
GASSER, H. S. 1930. Physiol. Reviews, x, 35.
HASAMA, BUN-ICHI. 1930. Arch. f. Exp. Path. u. Pharm., cliii, 129, 140.
1931. Ibid., clx, 92, 100, 107.
LAPICQUE, L. AND I. MEYERSON. 1912. C. R. Soc. Biol., lxxii, 63.
LEESE, C. E. AND L. EINARSON. 1932. This Journal, in press.
LINDHARD, J. 1931. Ergebn. d. Physiol., xxxiii, 337.
ORBELI, L. AND E. T. v. BRÜCKE. 1910. Pflüger's Arch., cxxxiii, 341.
RICHTER, C. P. 1924. This Journal, lxvii, 612.
ROSENBLUETH, A. 1932a. Ibid., ci, 149.
1932b. Ibid., cii, 12.
ROSENBLUETH, A. AND W. B. CANNON. 1932. Ibid., xcix, 398.
ROSENBLUETH, A. AND D. McK. RIOCH. 1933. Ibid., ciii, 681.
SAMOJLOFF, A. 1925. Pflüger's Arch., ccviii, 508.
SCHENCK, F. 1895. Ibid., lxi, 494.
STEWART, C. C. 1900. This Journal, iv, 185.
STÜBEL, H. 1912. Pflüger's Arch., cxliii, 381.

THE NATURE OF THE RESPONSES OF SMOOTH MUSCLE TO ADRENIN AND THE AUGMENTOR ACTION OF COCAINE FOR SYMPATHETIC STIMULI

A. ROSENBLUETH AND D. McK. RIOCH

From the Laboratories of Physiology and Anatomy in the Harvard Medical School

Received for publication October 19, 1932

In previous work by one of us (Rosenblueth, 1932b; Rosenblueth, Leese and Lambert, 1933) the assumption was made that the responses obtained from the excitation of smooth muscle by intravenous injections of adrenin are strictly comparable to those obtained by stimulation of the sympathetic nervous supply of the muscle. The second paper above mentioned furnishes evidence that the electrical potential which attends excitation and precedes the mechanical response is present when nerves are stimulated but is absent when adrenin is the stimulus. This raises the question whether the responses to adrenin must be considered as contractures (see Gasser, 1930) or whether they are properly contractions of the muscle involved. Indeed, Gasser (loc. cit.) defines contracture by the lack of excitation potentials, among other characteristics. He mentions, moreover, that contracture is attended by a refractory state of the muscle to nervous stimuli.

This latter criterion for contracture was adopted in order to test the nature of the responses to adrenin. Incidentally the results would show whether there exists a physiological synergy of secreted adrenin and simultaneous sympathetic nervous impulses.

The second problem mentioned in the title of this paper was suggested by the observation that the responses to sympathin are sensitized by cocaine (Rosenblueth and Schlossberg, 1931). If, then, contraction is due to the sympathin produced locally when the nerves are stimulated (Cannon and Bacq, 1931), the responses to stimulation of the nerves should increase after cocaine. The question reappeared in these experiments because cocaine was used to increase the responses to adrenin and the sympathetic was stimulated.

METHOD. The muscle chosen was the nictitating membrane of the cat. The responses were recorded as described by Rosenblueth and Cannon (1932). Supramaximal make and break shocks from a Harvard inductorium, timed by listening to a clock, were applied to the cervical sympathetic through shielded electrodes. The solutions of cocaine and adrenalin were injected intravenously. Dial anesthesia was used.

RESULTS. The effects on the membrane of stimulating the nerves at a given frequency, or of injecting a given dose of adrenalin, have been described elsewhere (Rosenblueth, 1932a, b). A dose of adrenalin and a frequency of nervous stimulation were chosen so that the heights of the responses (fig. 1-A and B) were at approximately the same point in the ascending branch of the corresponding hyperbolas (cf. the same papers).

If the nerves are stimulated at the peak of the response to adrenalin there occurs further contraction and the total height reached by the myograph is approximately that which would be obtained by doubling either the frequency of nervous stimulation or the dose of adrenalin injected

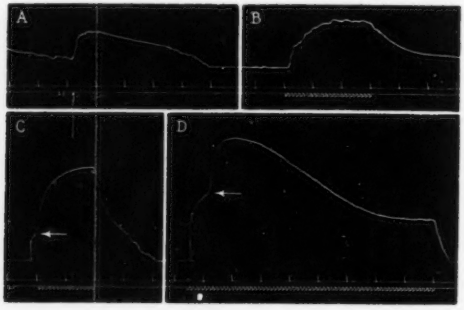


Fig. 1. Dial, 0.7 cc. per k. Electrodes on the cervical sympathetic. Time in 30-second intervals.

A. Adrenalin (1 cc. of 1:100,000) injected at signal.

B. Supramaximal make and break induction shocks at the frequency of 1 per 2 seconds.

C. Same dose of adrenalin as in A injected at the first signal; stimulation of the nerves 30 seconds later at the same frequency as in B. The arrow points to the beginning of action of the nervous impulses.

D. Stimulation of the nerves as in B. The lowest signal marks the injection of adrenalin. The arrow points to the beginning of action of the latter stimulus.

(fig. 1-C). If the stimulation is continued with the same frequency, the contraction diminishes at the rate at which the action of adrenin disappears and reaches a permanent level corresponding to the frequency used.

If the nerves are stimulated at a given frequency and adrenin is injected when the corresponding tetanic plateau has been reached, the typical curve for adrenin, slightly reduced in height, superimposes itself on the plateau. The muscle returns to this level when the action of the hormone is over (fig. 1-D).

The same observations repeated after about 8 mgm. per k. of cocaine have been injected intravenously give qualitatively similar results (fig. 2-A, C, D and E). The height of all the responses is, however, now

increased. The greater reaction to the same frequency of nervous stimulation (compare fig. 1-B with fig. 2-B) is as definite as the increase in the response to adrenin (fig. 1-A and fig. 2-A). The relaxation time is likewise increased by cocaine for either type of stimulation.

Discussion. From the results mentioned, summation of the responses occurring freely whatever the stimulus employed (figs. 1 and 2), it is clear that the muscle under the influence of adrenin is not refractory to nervous stimulation and *vice versa*. It may be safely asserted that we are dealing

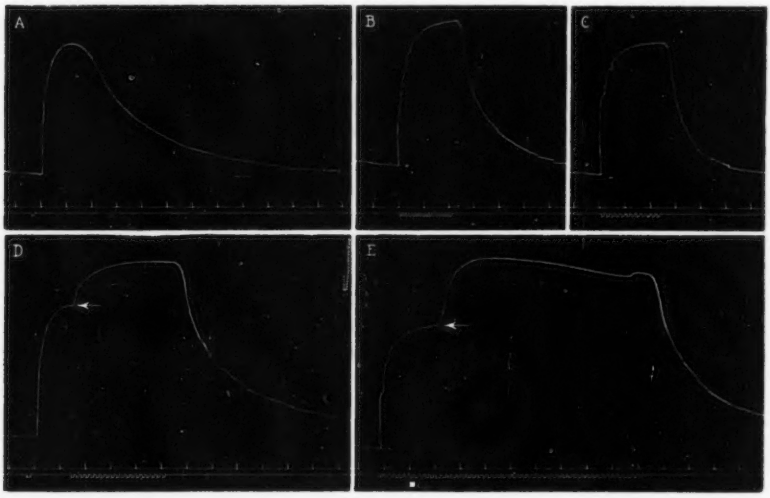


Fig. 2. Same preparation as in figure 1, but 8 mgm. of cocaine per k. injected intravenously.

A. Same as figure 1-A.

B. Same as figure 1-B.

C. Stimulation with a frequency of 1 shock per 3 seconds. This same frequency is used in records D and E.

D. Same as figure 1-C.

E. Same as figure 1-D.

with temporal and not spatial summation, for both types of stimulation used must affect all the cells in the muscle. The electrical stimuli applied to the nerve being supramaximal, all the nerves must be activated, and therefore all the muscle cells. Adrenin being carried by the blood stream must diffuse to all the elements.

Cannon (1914) has argued that when the sympathetic system is excited, it acts as a whole. During emotional excitement, for instance, the sympathetic discharges occur simultaneously in cervical sympathetic fibers and

in splanchnic fibers causing secretion of adrenin. Our results show that there will be a greater response of the effectors involved than if either activating agent were working alone and submaximally. It is pertinent to recall that Rosenblueth and Cannon (1932) found the effects of adrenin and sympathin to be synergical.

Since the muscle contracted by adrenin is not refractory to nervous impulses, it is concluded that the responses to adrenin are not contractures, but contractions, in all points comparable to those obtained from nervous discharges to the muscle, excepting for the lack of excitation potentials (Rosenblueth, Leese and Lambert, 1933). If Gasser's (loc. cit.) definition for contracture be accepted, our conclusion is not justified, because of this lack of excitation potentials. It is not a mere verbal distinction which is implied; it is important to discriminate between artificial or abnormal mechanical responses of muscle and true physiological processes.

The fact that cocaine augments the muscular contraction on nervous stimulation offers several interesting suggestions. The initial potentials were not found to be increased after cocaine (Rosenblueth, Leese and Lambert, loc. cit.). This agrees with the all-or-none character of the electric responses in muscle (Adrian, 1922). There appears then an independence of the mechanical response from the electrical disturbance. This is in accord with the same independence found when analyzing the action of adrenin and ergotoxine on the electrical potentials (Rosenblueth, Leese and Lambert, loc. cit.).

Since the amount of mediator produced per stimulus has been assumed to be constant (Rosenblueth, 1932b), and the electrical disturbance has likewise been found not to vary after cocaine, although the mechanical response increases, the interpretation that the excitation potential is related to the production of the mediator (Rosenblueth, Leese and Lambert, loc. cit.) is confirmed by these data.

The sensitizing action of cocaine both for the responses to nervous stimulation and for the responses to adrenin occurs then probably at the effector, either by increasing the responsiveness of the contractile system, or by diminishing the rate of destruction of adrenin and sympathin *in situ*, or finally, by catalyzing the combination of the hormones with the substances in the muscle.

SUMMARY AND CONCLUSIONS

The summation of responses to adrenin and simultaneous sympathetic nervous impulses, and the effects of cocaine on the contractions evoked by nervous stimulation were studied in the nictitating membrane of the cat.

Summation of the responses of the muscle to simultaneous action of both types of stimuli occurs freely (figs. 1 and 2). It is therefore concluded that the muscle under the influence of adrenin is not refractory

to nervous impulses. This leads to the conclusion that the responses to adrenin are contractions and not contractures, notwithstanding the lack of excitation electrical potentials when adrenin stimulates.

Cocaine increases the contractions obtained by nervous stimulation (compare figs. 1 and 2), although it does not affect the electric responses.

BIBLIOGRAPHY

ADRIAN, E. D. 1922. Arch. Neer. de Physiol., vii, 330.
CANNON, W. B. 1914. This Journal, xxxiii, 356
CANNON, W. B. AND Z. M. BACQ. 1931. Ibid., xcvi, 392.
GASSER, H. S. 1930. Physiol. Reviews, x, 35.
ROSENBLUETH, A. 1932a. This Journal, ci, 149.
1932b. Ibid., cii, 12.
ROSENBLUETH, A. AND W. B. CANNON. 1932. Ibid., xcix, 398.
ROSENBLUETH, A., C. LEESE AND E. LAMBERT. 1933. Ibid., ciii, 659.
ROSENBLUETH, A. AND T. SCHLOSSBERG. 1931. Ibid., xcvii, 365.

STUDIES ON THE ADRENAL

I. Adrenalectomy in Mammals with Particular Reference to the White Rat (Mus norvegicus)[1]

W. M. FIROR and ARTHUR GROLLMAN[2]

From the Departments of Surgery and Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine

Received for publication October 27, 1932

During the course of studies on the nature of the cortical hormone of the adrenals we have accumulated data on the survival of various mammalia after adrenalectomy which bear on the discordant data of previous observers. Such discrepancies have been attributed in the past chiefly to variations in the thoroughness of the surgical technique employed. However, adrenalectomy in the hands of very proficient surgeons often leads to results (insofar as survival of the experimental animal is concerned) which are far inferior to those obtained by less experienced or skillful operators. In addition to surgical technique the following factors have been found to determine the length of survival of an adrenalectomized animal in the case of the mammals which we have studied: 1, the animal species; 2, the age of the animal; 3, the pre-operative treatment; 4, the anesthesia and its duration; 5, the completeness of the extirpation; 6, the post-operative treatment.

The rat (*Mus norvegicus*). The effect of adrenalectomy in the white rat is of practical importance since its size renders it convenient for the standardization of extracts of cortical hormone. In recent years it has been generally assumed that adrenalectomy in rats results in a mortality of only about 50 per cent. Thus Exner (1920), Mauerhofer (1922), Scott (1923), Lewis (1923), Durrant (1924), Rogoff and Necker (1925), Marine and Baumann (1927), Perla and Gottesman (1931), Britton (1931), von Árvay and Verzár (1931), Hartman and his collaborators (1931), and many others state that these animals are capable of long survival and differ from other mammals in this respect. Marti (1923) claimed that rats survived adrenalectomy even after removal of the accessory adrenal bodies. Opposed to this conception is the work of Jaffe (1926) and Pencharz, Olmsted and Giragossintz (1931). Jaffe found that of 90 rats 35 per cent died within thirty days, the majority before

[1] A preliminary report of the results presented in this paper was given at the annual meeting of the Federation of American Societies for Experimental Biology, Philadelphia, 1932.

[2] Aided by a grant from the Hartley Corporation, for which we wish to express our appreciation.

the thirteenth day, of acute adrenal insufficiency. Forty-six per cent showed chronic insufficiency and died within seven months without the presence of gross accessories being manifested at autopsy. The remaining 19 per cent were unaffected by the operation but had large accessory cortical tissue at autopsy. Pencharz, Olmsted and Giragossintz (1931) proceeded further in their analysis and concluded that the rat is no exception to the rule that insufficiency and death result in rats deprived of their adrenals. In 62 rats death occurred in from two to eighteen days with no survivals. If only 5 per cent of the gland was left in the operation, rapid regeneration occurred and the animal survived. Martin (1932) observed death in 80 per cent only of 121 operated animals. Of the 24 survivals, cortical tissue was observed at autopsy in only 11. Kutz (1931) found that 56 of a total of 57 rats adrenalectomized at the age of twenty-eight days had died by the tenth day. Freed, Brownfield and Evans (1931) also state that adrenalectomy is fatal if a quarter of an inch of pedicle is removed with the gland. Gaunt (1932), on the other hand, found that rats which he classes as belonging to a "T-strain" survive adrenalectomy.

Despite all the work cited above there thus remains disagreement as to the effect of adrenalectomy on the white rat. In the present study we report observations on over 250 adrenalectomized rats. In order to settle the problem of whether a rat can survive without the presence of accessory cortical tissue, and to determine to what extent such cortical tissue occurs and is capable of prolonging life, we have sectioned and microscopically examined all animals in which prolonged survival made apposite the question of the functional capacity of accessory bodies or the possibility of an incomplete operation.

METHODS. The animals were for the most part the ordinary laboratory albino variety (of the Wistar strain), some raised for several generations in this laboratory and others obtained from various dealers. In order to eliminate the possibility of different strains reacting differently we have used animals from different sources and included piebald varieties as well as some wild rats (Mus norvegicus). The animals were kept on a mixed diet in standard aluminum cages. Care was taken to adjust the temperature of the room. In this way the common respiratory infections which often cause a high mortality even in unoperated animals were avoided. The mortality rate in our unoperated animals less than one year in age was negligible.

All operations were carried out in one stage under light ether anesthesia. The operative field was shaved and prepared with 70 per cent alcohol. A single transverse incision was made in the skin of the lumbar region. The muscles in one costovertebral angle were divided parallel to the lowermost rib and posterior to the lumbar vein. The loose tissue medial to the adrenal gland was grasped with fine curved artery forceps. The tissues attached to the gland were incised distal to these forceps and the gland was lifted out. The defect in the lumbar muscles was closed with a single suture. The rat was then turned and a similar procedure carried out on the opposite side, and the skin sutured.

At no time during the operation was either adrenal gland picked up with forceps. The criterion of a satisfactory operation was not merely that the capsules of the glands be intact but that each gland be removed with an appreciable amount of adjacent tissue. In large rats the bulk of this

tissue was three or four times that of the adrenal gland. In the smallest rats this tissue was merely a thin veil of peritoneum and blood vessels. It is in this areolar and connective tissue that cellular rests or aberrant microscopic accessory cortical glands exist which in the absence of the chief gland hypertrophy. Rats in which the operation was unsatisfactory according to the above criteria were especially marked, and it was found that this group comprised the ones with an apparently indefinite survival period, to be discussed later.

Symptomatology of adrenalectomized rats. Adrenal insufficiency in rats is characterized by many of the symptoms noted in other mammals. The loss of weight accompanying the anorexia characteristic of adrenal insufficiency is very pronounced, and serves as a valuable criterion of the adequacy of replacement therapy. Another important symptom of insufficiency which serves, together with the loss of weight, as a test of the efficacy of a replacement therapy is the change in body temperature, which drops from a normal value of about 100°F. to less than 92° before death.

A rat in insufficiency manifests a typical appearance. It sits quietly with its back hunched and fur ruffled, and is disinterested in its surroundings. Rats rarely manifest the pyogenic infections at the site of operation which are observed in cats or dogs. This is characteristic of their general freedom from pyogenic infections. In marked insufficiency, however, an infection of the eyes, and in male rats priapism, both of which are possibly pyogenic in origin, frequently occur. Persistent diarrhea, frequent in dogs, also occurs at times, with occasional bloody diarrhea due to intestinal hemorrhage. Final collapse, labored respiration, and convulsions are premortem symptoms similar to those observed in larger mammalia.

The absence of normal sexual activity in the adrenalectomized rats is very marked. Pathological changes in the testis and interference with the normal oestrous cycle have been demonstrated by many observers (more recently by Freed, Brownfield and Evans, 1931; Martin, 1932; Atwell, 1932, and others). We have repeatedly confirmed this in our own series. In over 200 animals no pregnancies occurred after operation. The normal breeding of rats and mice reported by Lewis (1923) and Deanesly (1928) are attributable to the incompleteness of their double adrenalectomy as evidenced by the high percentage of survival observed by these workers.

Effect of age on survival period. In table 1 are collected data for the survival period of 158 adrenalectomized rats. It will be seen that the age of the rat is a predominant factor in determining the survival period. With increasing size (*i.e.*, age) the survival period increases, rats weighing less than 50 grams surviving on an average only one day, those weighing over 200 grams surviving on an average nine days. As will be shown later, apparent indefinite survival is due to incomplete operation and hyper-

trophy of cell residues or small accessory bodies. It requires some days for these to assume a size sufficiently large to maintain life, and consequently this can occur only in older animals in which survival extends, even with complete removal, for a week or more. The older the rat, the more likely are such residues to hypertrophy and permit indefinite survival. This has led some previous observers to the conclusion that adrenalectomy is fatal only in very young rats. The relatively larger size of the adrenal glands in young animals speaks for the greater demand of the organism for the cortical secretory product during earlier life.

Our data recorded in table 1 are found to be in excellent agreement with the results of Pencharz, Olmsted, and Giragossintz (1931) if we tabulate the results of these authors on 62 rats on the basis of weight as in table 1. In 24 animals weighing 101 to 150 grams these authors found an average

TABLE 1

The survival period of 157 double adrenalectomized untreated rats. Operation performed under ether anesthesia at one sitting

WEIGHT OF RAT	NUMBER OF DAYS SURVIVING																			TOTAL NUMBER OF RATS STUDIED	AVERAGE SURVIVAL PERIOD
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
grams																							*days*
Less than 50	6																					6	1.0
50 to 75	7	3	2	1	1																	14	2.0
76 to 100	7	3	4	5	6	6	4	2	1													38	4.3
101 to 150	4	2	3	4	6	7	7	2	2	2	1	1	2	1		3		1				48	7.0
151 to 200	3	1	2	1		2	2		2		1				1	1	1		2	1		20	8.6
Over 200	0	3	1	5	2	3	2	3	1	2	1	2	1	1		1			1	2	1	32	9.0

survival period of 7.9 days as compared to 7.0 days in our own series. In 19 rats weighing 151 to 200 grams their survival period is 9.9 days as compared to 9.0 in our series.

In the case of the rats of table 1 which survived longer than seventeen days microscopic sections of the site of operation were made, but no evidence of any adrenal tissue was found. It is improbable, in view of the general distribution of the results in table 1, that microscopic rests of tissue which failed to hypertrophy may have prolonged the life of these animals.

Apparent indefinite survival of adrenalectomized rats. From table 1 have been excluded a group of 22 other animals which survived over twenty days and in all of which microscopic sections demonstrated the existence of glandular tissue of a type more or less characteristic of the adrenal cortex. In the majority of these 22 animals the incompleteness of the operative removal was noted at the time of operation and was due to inadvertent breaking of the gland or the failure to excise completely the

surrounding veil of connective tissue. The prolongation of life in these 22 rats was due to the presence of physiologically active glandular tissue. These rats represent 12 per cent of all operated rats weighing over 100 grams at operation. These 22 surviving rats were clinically divisible into two groups. About half of them continued to grow and act normally after several days' recuperation from the operation. In such animals well developed glandular tissue was found on reoperation. These glands were normal in size, and histologically, except for the absence of medullary tissue, showed the same cellular arrangement as is found in the normal adrenal cortex. In one case the gland contained tissue having the histological appearance of the X-zone, described by Howard (1927) in the mouse, but not previously reported in the rat. After removal of the regenerated glands at a second operation, the usual signs of adrenal insufficiency ensued and death resulted in a period of time in conformity with the results of table 1.

The remaining half of the 22 rats not included in table 1 survived for periods of from 19 to 50 days with slow loss of weight and eventual manifestations of the signs of adrenal insufficiency and ultimate death. These animals thus differed from those enumerated in table 1 by manifesting a less intense and more chronic form of cortical insufficiency. In these animals, rests of glandular cortical tissue were found on histological examination of sections at the site of operation. This tissue was very small, however, in comparison with that found in animals surviving indefinitely without signs of insufficiency. Moreover, the cells often did not show the normal histological arrangement and were at times vacuolated. In this second group of rats there was a failure of the tissue to hypertrophy to form a new and normal gland, with the resulting insufficiency and ultimate death. It is probable that failure to hypertrophy is due to an inadequate blood supply to the area in which the cell fragment remains. Such tissue is often found on the surface of a lymph gland into which the cortical tissue infiltrates.

In none of the rats of our series has it been necessary to invoke the presence of active cortical tissue in regions of the body other than at the site of the adrenal bodies to explain long survivals after adrenalectomy. The presence of accessory adrenal tissue in the reproductive organs and other areas has been assumed by many; but our results would indicate that such accessory bodies are either chromaphil tissue or, if cortical, are unable to hypertrophy and assume the functional capacity of the adrenal cortex.

The relatively high percentage (12 per cent) of our total rats in which the original operation was incomplete was gradually reduced with practice in the operative technique. At present we encounter a rat in which later examination reveals the presence of hypertrophied glandular tissue in only about 5 per cent of operated animals.

Pre-operative treatment. The pre-operative care is of utmost importance in determining the survival period after adrenalectomy. Animals obtained from sources in which temperature conditions, food, etc., were not optimal succumb much more quickly than those previously kept for some time under good laboratory conditions and in a good state of health and nutrition. This is best illustrated by a series of cats which were subjected to cold before the removal of only one adrenal (the right). Such animals, as is well known, normally survive unilateral adrenalectomy without manifesting any signs of insufficiency. The six cats of this series were subjected pre-operatively to a room temperature of 10°C. for several days. All showed signs of adrenal insufficiency, and in 4 out of 6 of such animals death ensued within ten days. In another simultaneous series of control animals no signs of insufficiency ensued nor did death occur in any case.

The effect of the anesthetic. The fact that the operative technique is an important factor in determining the survival of adrenalectomized animals has been often emphasized. The shock of trauma and hemorrhage involved in prolonged and clumsy handling of the viscera will obviously shorten the life of an already doomed animal. Anesthesia also plays a very important rôle in determining the survival of an animal, so that the celerity of the operation is of great importance, particularly with ether anesthesia.

The effect of the length of time during which the ether is administered is shown in the following series of 10 rats of approximately equal weights (200 to 220 grams). The average survival of 5 rats receiving ether for five minutes was fourteen days, while in 5 receiving ether for ten minutes the average survival period was only 7.3 days or only half of that in the first group. Prolonged administration of ether thus reduced to half the survival period. In another series of 8 rats in which the presence of excessive adipose tissue rendered adrenalectomy difficult and prolonged the period of anesthesia to ten minutes instead of about five minutes as usual, the average survival period was 6.1 days with a minimum of 4 days and a maximum of 10 days. The rats weighed over 250 grams, the average survival of which after a quicker operation and shorter period of anesthesia would have been much longer (see table 1).

The deleterious effects of ether in reducing the survival period of adrenalectomized animals is also shown in dogs in which the results of general anesthesia under ether were compared to those of spinal anesthesia. In a series of 8 dogs, bilateral adrenalectomy was performed in one stage. The first group of 4 animals in which ether was used quickly recovered from the effects of the operation and anesthesia. They showed no evidence of shock and were apparently normal during the remainder of the operative day, but each of these animals was found dead on the following morning. Necropsy failed to show any explanation for their sudden death except

the complete absence of the adrenals. In contrast to the foregoing were the survival periods of the other 4 dogs in which a similar operation was performed under identical conditions except for the use of a spinal anesthetic instead of ether. These results are reproduced in table 2.

The last two dogs cited in this table were not allowed to die but were given cortical extracts after 72 and 96 hours respectively, when signs of insufficiency appeared. Thus it is seen that with spinal anesthesia the average survival period was over 97 hours, whereas with ether the average was less than 21 hours. In our subsequent work on dogs we have, therefore, resorted exclusively to the use of spinal anesthesia with markedly superior results to those of our earlier work with ether.

To avoid the deleterious effects of ether in reducing the survival period after adrenalectomy we have used amytal[3] (sodium iso-amyl-ethyl barbiturate) in subsequent work on rats, injecting 0.1 cc. of a 10 per cent solution per 100 grams of body weight, intraperitoneally. In rats in which this

TABLE 2

Adrenalectomy in dogs, using spinal anesthesia

BREED	DOSAGE OF ANESTHETIC	SURVIVAL
Chesapeake Bay	200 mgm. novocain	76 hours
Collie	100 mgm. tutocain	144 hours
Collie	140–150 mgm tutocain	Over 72 hours
Fox terrier	60 mgm. tutocain	Over 96 hours

dosage was insufficient 30 per cent more was injected after fifteen minutes. This anesthesia has markedly prolonged the survival period of our rats beyond the times shown in table 1.

One may obtain a measure of the effect of the operative interference (including the action of the anesthetic) in reducing the survival period of an animal in the following manner: By efficacious therapeutic treatment[4] the animal may be restored to normal and allowed to recover from the operative wound and resume its normal growth and activity. If treatment in such an animal now is suddenly stopped, it will be found that survival extends for a longer period than would have been observed had the animal not been treated. We may consider the difference between the observed survival period after cessation of therapy of a series of treated animals and a similar series allowed to die untreated as representing the period of life shortened by the operation. Thus the average survival period of a series of rats (weighing 76 to 100 grams) was 6.3 days after cessation of injection

[3] We are indebted to the Eli Lilly Co. for generous supplies of amytal.

[4] The method of preparation of our extracts will be described elsewhere. For a preliminary report *cf.* Grollman and Firor (1932).

of a potent extract over a period of two weeks as compared to 4.3 days (see table 1) in the non-treated series. Similarly a group of rats weighing 101 to 150 grams survived on an average 10.2 days as compared to 7.0 in the untreated series. We may consider the differences in these survival periods as representing the operative effect—chiefly of the anesthetic—in draining the organism of its supply of cortical hormone.

The above described prolonged survival period of treated animals after cessation of treatment has also been observed by Swingle and Pfiffner (1931) in cats. The question arises, however, as to the possibility of cellular remnants left at operation hypertrophying during the treatment and thus accounting for the very prolonged survival (in one case 60 days) observed by these authors. Although such remnants might be undetected by the usual macroscopic necropsy, they may suffice to prolong life for some time. In our own series of animals quoted above we have sectioned the site of operation, and in animals surviving for prolonged periods have always found such remnants. An animal which if left untreated would die in the time cited in table 1, will after several weeks' treatment often survive indefinitely after the cessation of the treatment. In such animals we have always found regenerated cortical tissue on microscopic examination. This fact must be borne in mind to avoid exaggerating the therapeutic efficacy of a given preparation. During the course of treatment time is allowed for regeneration of active tissue which later supplants the necessity of administering hormone which, in a completely adrenalectomized animal, would still be required.

Pregnancy. The effect of pregnancy on the survival period of adrenalectomized animals has been previously investigated with contradictory results. H. A. Stewart (1912) first noted the prolongation of the survival period of adrenalectomized pregnant cats as compared to non-gravid females. G. N. Stewart and Rogoff (1927) in their study on dogs also found a significant prolongation of life in pregnant animals. Corey (1928), on the other hand, found no effect of pregnancy or lactation on survival in cats, and Carr (1931) also reports no effect of pregnancy in the white rat.

In our series of rats we have found a definite prolongation of life in adrenalectomized pregnant rats as compared to non-gravid females of the same size. The results with 10 pregnant animals are given in table 3, from which are excluded 4 other rats surviving over twenty days in which remnants of cortical tissue were found on microscopic section of the adrenal site. The average survival period was 15.4 days, which is almost twice as long as the average of all non-pregnant animals (see table 1). Contrary to Carr's results we thus find a marked effect of pregnancy on prolonging the survival period in the white rat. Of the above mentioned 10 rats, 6 delivered normal litters and mothered them in most cases for several days. In each case, however, the young died of lack of nutrition

due to the absence of milk. This absence of milk is possibly due to dysfunction of the mammary gland caused by lack of the cortical hormone, similar to the insufficiency manifested by the kidney, liver, muscles, etc. It may also be attributed to an interference with normal pituitary or other glandular functioning necessary to maintain mammary activity. In the case of the 4 rats which died in incipient labor, it is possible that the strain of delivery contributed to the cause of death. In no case, on the other hand, have we observed death in an adrenalectomized rat during the earlier stages of a grossly evident pregnancy.

WHITE MICE (MUS MUSCULUS). The effect of adrenalectomy on white mice appeared to be of interest because the size of this animal offered the

TABLE 3

Survival of pregnant rats following adrenalectomy

RAT NUMBER	TOTAL DAYS SURVIVING OPERATION	DAYS OF PREGNANCY FOLLOWING OPERATION	DAYS SURVIVING POSTPARTUM
26	20	20	Died in incipient labor
163	13	13	Died in incipient labor
219	18	14	4
234	21	21	Died in incipient labor
236	11	1	10
238	17	1	16
239	17	1	16
240	15	1	14
258	15	8	7
261	7	7	Died in incipient labor
Average.....	15.4	8.7	11.1*

* 6.7, if the rats dying undelivered are included in the average.

same advantage over the rat as the latter over the larger mammals. Moreover, the mouse is not without specific interest because of certain characteristics in the histological development of its adrenals which render it more analogous to the human than other mammals (Howard, 1927).

The small size of the gland made complete removal often difficult. From the standpoint of operative technique the mouse, therefore, proved itself to be inferior to the rat. The animals were all standard laboratory white mice, weighing between 15 and 20 grams. The operative procedure was identical with that used in the rat. Of a total of 20 animals the following survival periods were observed:

Number of mice.....	4	4	1	4	1	1	1	1
Survival in days.....	0	1	3	5	7	9	10	11

The average survival period of the above listed 13 animals which survived the operation was thus 5 days. If we exclude the animals surviving only

one day, in which no doubt the hemorrhage and operative shock may be considered as the prime cause of death and which might be avoided with further practice in the technique, the average survival period was 6.7 days. These animals showed the same subjective symptoms as were observed in rats. The gradual weight loss is indicated in the following mouse, the daily weights of which during 10 days of survival were 17.7, 17.7, 17.5, 17.0, 16.1, 15.1, 16.0, 16.3, 16.2, 15.7 grams respectively.

Of the remaining three mice, one survived 38 days, during which time it gained only 3 grams, indicating insufficiency. The site of operation was sectioned and found to contain, when microscopically examined, a small area of cortical glandular material. The two others manifested, after recovery from the operation, no signs of adrenal insufficiency, and were sacrificed on the 43rd day of survival. One had a well formed adrenal gland which had regenerated from cells left at operation. The second mouse also had a mass of atypical cortical glandular cells which apparently had sufficed to maintain it in a state of good health and nutrition.

White mice thus react to adrenalectomy in the same way as do rats, and may be used in experimental studies where the smaller animal is desirable.

THE RABBIT. The rabbit, like the rat, is generally considered as an animal in which widespread occurrence of accessory cortical tissue, in at least 25 per cent of all cases, permits indefinite survival after adrenalectomy. Kahn (1917), Mauerhofer (1922), Rogoff and Dominguez (1924) Marine and Baumann (1927), Baumann and Kurland (1927), Ohguri (1931), Oikawa (1931), and others support this view. Certain investigators, however, including Hultgren and Andersson (1899), Strehl and Weiss (1901), Kosch (1914), Reiss (1930), and Magistris (1932) found adrenalectomy, if performed in a one-stage operation, to be always fatal to rabbits except in rare cases where accessory organs at the site of operation could be demonstrated at autopsy. Our own results confirm this view and indicate that as in the rat, monkey,[5] cat, and dog, adrenalectomy, if complete, will not permit survival and that the occurrence of survival is usually due not to the presence of widely distributed accessory glands but to the regeneration of bits of tissue left at the site of operation, particularly along the vena cava, to which the right gland is normally closely adherent. If done in two stages ample time elapses for the regeneration of scattered microscopic rests which, after the removal of the second gland, are sufficient to permit apparently normal adrenal function. This we believe explains the much more frequent survival observed after the two-stage as

[5] The discrete anatomical structure of both of the adrenal glands in the monkey renders them easily amenable to complete removal. The operation is much easier than in the dog or cat. Our results on six animals (macacus rhesus) corroborate the previous findings of Kahn (1912).

compared to the one-stage operation, although the other facts already outlined above in connection with the rat must also play a part.

Operative technique. A high rectus incision about four inches long was made to the right of the midline. The left adrenal was exposed and the peritoneum surrounding it was incised. Curved mosquito forceps were placed posterior to the gland, and a ligature of silk was securely tied about the areolar tissue and blood vessels distal to the forceps. This tissue was then divided between the ligature and the clamps. By carefully placing the forceps so as not to encroach upon the gland and by keeping these forceps in place while the adjacent tissue was ligated and divided, a complete ablation of cortical tissue is assured. On the right side the same principles were observed. Here, however, the proximity of the vena cava often necessitated including a portion of the posterior wall of this vessel in the forceps and ligature.

We have found a great operative mortality in rabbits, approximately half succumbing to the effects of the operation. The use of spinal anesthesia, which as we have seen greatly prolongs survival in the dog, had only a slight or no ameliorative effect. Neither did the use of amytal instead of ether.[6] Diarrhea was the most constant finding in early death of rabbits after operation. Occasionally premortem convulsions were observed.

In a series of rabbits which recovered from the operation the following survival periods were noted:

Number of rabbits.....	13	8	2	1	1	2	3	1	1	1	1	1
Survival period in days..	Less than 1	1	2	3	4	6	7	9	11	18	65	68	Over 120

Ninety per cent of the animals survived 11 days or less. In these there were the usual manifestations of adrenal insufficiency,—progressive loss of weight, subnormal temperature, anorexia, and a liability to suffer death from apparently minor causes. The act of catching the rabbit would often throw it into convulsions similar to those seen in the hypoglycemia after insulin injection. All the rabbits were autopsied and serial sections made of all suspicious residue in the vicinity of the normal site of the adrenal glands. In the rabbits surviving 9 days or less no adrenal tissue was demonstrable. In the rabbit surviving 11 days no adrenal tissue was seen at autopsy, but microscopic examination revealed a small piece of typical adrenal cortex. The rabbit surviving 18 days had a small bit of adrenal tissue in the peritoneal fascia. In the rabbits surviving 65 and 68

[6] It is noteworthy that Kahn (1917), who reports few fatalities in rabbits, used no anesthetic.

days a glandular mass the size of a normal adrenal gland was found. These had not been present at the first operation and represented, therefore, an active regeneration of tissue left at operation. In the rabbit surviving 120 days a well formed cylindrical mass of cortical tissue about 3 mm. in diameter and 6 mm. long was found at autopsy in the interior of the abdominal vena cava. Whether this was its original site or whether it developed from cellular masses accidentally implanted during resection of the vessel is questionable.

Our results would indicate that the generally accepted view regarding the frequency of the existence of scattered cortical tissue in the rabbit is based on incomplete removal. Complete removal is difficult; but it is unnecessary to assume that tissue capable of supporting life exists along the vena cava, and diffusely scattered throughout the dorsal peritoneal spaces or in the reproductive organs, as claimed by Ohguri (1931) and others. In no case have we found such tissue or been forced to assume its existence in order to explain survival of an adrenalectomized animal. Furthermore, our findings explain the apparently paradoxical effect of castration on the survival of adrenalectomized mammals. Such effect would obviously be inconsistent with the presence of accessory glands in the region of the sexual glands.

SUMMARY

The rat and the rabbit are no exception to the rule that adrenalectomy is fatal to mammalia. Survival is due to incomplete extirpation, which consists either in allowing cortical remnants of the main glands to be left behind, or in failing to remove the scattered accessory nests of cells present in the peritoneal tissue in the neighborhood of the glands. In every case of indefinite or prolonged survival, microscopic examination has revealed the presence of hypertrophied cortical tissue in this region. The assumption of the existence of widespread accessory tissue is unnecessary. Factors influencing the survival of adrenalectomized animals are described, such as the effects of pre- and post-operative care, the nature of the anesthetic, the age of the animal, and pregnancy. The use of a spinal anesthetic instead of ether markedly prolongs the life of adrenalectomized dogs. Young rats may be used for the assay of cortical extracts.

BIBLIOGRAPHY

VON ÁRVAY, A. AND F. VERZÁR. 1931. Biochem. Zeitschr., ccxxxiv, 186.
ATWELL, W. J. 1932. Proc. Soc. Exper. Biol. and Med., xxix, 1259.
BAUMANN, E. J. AND S. KURLAND. 1927. Journ. Biol. Chem., lxxi, 281.
BRITTON, S. W. 1931. This Journal, xcix, 9.
CARR, J. L. 1931. Proc. Soc. Exper. Biol. and Med., xxix, 128.
COREY, E. Z. 1928. Physiol. Zool., i, 147.
DEANSLEY, R. 1928. Proc. Roy. Soc. of London, B. ciii, 523.

DURRANT, E. P. 1924. This Journal, lxx, 344.
EXNER, H. V. 1920. Dublin Journ. Med. Sci., series 4, i, 79.
FREED, S. C., B. BROWNFIELD AND H. M. EVANS. 1931. Proc. Soc. Exper. Biol. and Med., xxix, 1.
GAUNT, R. 1932. Proc. Soc. Exper. Biol. and Med., xxix, 823.
GROLLMAN, A. AND W. M. FIROR. 1932. This Journal, ci, 46.
HARTMAN, F. A., B. D. BOWEN, G. W. THORN AND C. W. GREENE. 1931. Ann. Int. Med., v, 539.
HOWARD, E. 1927. Amer. Journ. Anat., xl, 251.
HULTGREN, E. O. AND O. A. ANDERSON. 1899. Skand. Arch. f. Physiol., ix, 73.
JAFFE, H. L. 1926. This Journal, lxxviii, 453.
KAHN, R. H. 1912. Pflüger's Arch., cxlvi, 578.
1917. Pflüger's Arch., clxix, 326.
KAHN, R. H. AND E. STARKENSTEIN. 1911. Pflüger's Arch., cxxxix, 181.
KOSCH, O. 1914. Arch. f. exper. Path. u. Pharm., lxxvii, 432.
KUTZ, R. L. 1931. Proc. Soc. Exper. Biol. and Med., xxix, 91.
LEWIS, J. T. 1923. This Journal, lxiv, 503.
MAGISTRIS, H. 1932. Biochem. Zeitschr., ccxlviii, 39.
MARINE, D. AND E. J. BAUMANN. 1927. This Journal, lxxxi, 86.
MARTI, H. 1923. Zeitschr. f. Biol., lxxvii, 181.
MARTIN, S. J. 1932. This Journal, c, 180.
MAUERHOFER, E. 1922. Zeitschr. f. Biol., lxxiv, 147.
OHGURI, M. 1931. Tohoku Journ. Exper. Med., xvii, 349, 390.
OIKAWA, K. 1931. Tohuku Journ. Exper. Med., xviii, 1.
PENCHARZ, R. I., J. M. D. OLMSTED AND G. GIRAGOSSINTZ. 1931. Physiol. Zool., iv, 501.
PERLA, D. AND J. M. GOTTESMAN. 1931. Proc. Soc. Exper. Biol. and Med., xxviii, 650.
REISS, M. 1930. Endocrinol., vi, 321.
ROGOFF, M. AND R. DOMINGUEZ. 1924. Journ. Met. Res., vi, 141.
ROGOFF, M. AND J. DE NECKER. 1925. Journ. Pharm. Exper. Therap., xxvi, 243.
SCHWARZ, O. 1910. Pflüger's Arch., cxxxiv, 259.
SCOTT, W. J. M. 1923. Journ. Exper. Med., xxxviii, 543.
STEWART, G. N. AND J. M. ROGOFF. 1927. This Journal, lxxix, 508.
STEWART, H. A. 1912. Int. Cong. Med., xvii, section 3, p. 173.
STREHL, H. AND O. WEISS. 1901. Pflüger's Arch., lxxxvi, 107.
SWINGLE, W. W. AND J. J. PFIFFNER. 1931. This Journal, xcvi, 164.
WIESEL, J. 1898. Centralbl. f. Physiol., xii, 780.

THE EXCRETION OF IODINE IN EXPERIMENTAL HYPERTHYROIDISM

B. O. BARNES[1]

From the Physiological Laboratories of the University of Chicago

Received for publication October 24, 1932

The extensive literature on iodine metabolism has recently been reviewed by Fellenberg (1), Krayer (2), and Asimoff and Estrin (3). A few papers are closely related to the present work and must be discussed. Kendall (4) in 1919 reported that after the intravenous injection into a dog of 200 mgm. of thyroxine, 43 per cent of the iodine appeared in the bile and 13 per cent in the urine within 48 hours. Fellenberg (1) fed a single dose of beef thyroids to five normal persons and collected their urine for the following 24 hours. The amount of iodine recovered in the urine varied from 16 to 28 per cent of the total. Krayer (2) followed the iodine elimination in bile and urine from rats and guinea pigs under anesthesia. He found that after injecting rats with thyroxine, about 47 per cent of the iodine was eliminated in 3 hours in the bile, but after this time very little more was excreted. He was able to find iodine in the urine of only two out of ten rats injected with thyroxine. Asimoff and Estrin (3) fed two dogs a single dose of 100 and 75 grams of desiccated thyroids respectively. In 24 hours they recovered 25.1 per cent of the iodine in the urine and 1.7 per cent in the bile from the first dog. The second dog excreted 35.1 per cent in the urine and 1.69 per cent in the bile within 30 hours. The same authors (5) fed 61 mgm. of thyroxine by stomach tube to each of two dogs. They recovered 4.51 per cent and 8.54 per cent respectively in the urine and 10.0 per cent and 6.66 per cent in the bile. About 80.0 per cent was eliminated in the feces. The present work was almost complete before the papers of Asimoff and Estrin appeared.

METHODS. Dogs were kept in metal cages suitable for urine collection and the specimens collected every 24 hours. The iodine estimations were carried out by the technic of Kendall (6). In the first series ten normal dogs were fed Armour's desiccated thyroid which was mixed with their usual diet of meat, bread, and bone meal. The dosage in most instances was one gram of thyroid per kilo body weight daily although for a short time a few dogs were fed 2 and 3 grams per kilo. In four of these dogs

[1] National Research Fellow in Physiology.

the desiccated thyroid was replaced for ten days with thyroglobulin prepared by Oswald's method. Five other dogs were thyro-parathyroidectomized and kept free from tetany by the subcutaneous administration of calcium gluconate. They were likewise fed 1 gram per kilo of body weight of desiccated thyroid. In 14 additional dogs the common bile duct was ligated and a metal cannula inserted in the gall bladder. The cannula was brought through a stab wound and the bile collected in a rubber bag. Seven of these dogs were fed desiccated thyroid in doses of 1 to 2 grams per kilo, one receiving a single dose of 100 grams. The other seven were injected with thyroxine (Roche crystalline synthetic), some with a single dose while others received as many as 5 injections.

RESULTS. There was no difference observed between the percentage of total iodine eliminated in the urine when desiccated thyroid or thyroglobulin was fed. Increasing the dose had no effect on the percentage of urinary iodine. In table 1 the results from normal and thyroidectomized dogs are summarized.

TABLE 1

Showing the duration of thyroid feeding and the iodine eliminated in the urine

NORMAL DOGS			THYROIDECTOMIZED DOGS		
Dog	Days	I_2 (per cent of total fed)	Dog	Days	I_2 (per cent of total fed)
1	10	50.0	11	8	51.5
2	4	57.0	12	6	41.0
3	4	37.0	13	5	42.5
4	9	37.0	14	7	46.0
5	9	45.0	15	6	47.0
6	10	44.0			
7	24	51.5			
8	26	58.0			
9	92	57.5			
10	92	60.0			

The values range from 37 to 60 per cent of the total ingested, the lower values being obtained when the duration of the thyroid feeding was shorter. However, when the experiment was more prolonged between 50 and 60 per cent of the iodine fed was recovered in the urine. Undoubtedly one of the factors is the saturation of the tissues with the iodine compounds and this is more important in the shorter experiments. This would explain the low values which Fellenberg observed in his patients after a single dose. Results similar to his have been obtained on dogs the first day of the experiment. The range of the thyroidectomized dogs is within the limits of the normal animals although a little below the average. This may be explained in part by the brevity of the experiments.

If some iodine is taken up by the tissues, increasing the urine output should increase the iodine elimination. This has been observed frequently. If a dog developed polyuria for a few days, the urinary iodine was increased for one or two days and then became normal again. Dog 10, a pregnant animal, may be cited as a typical case. On the 25th day of the experiment she developed a marked diuresis which lasted for over a week when abortion occurred. The iodine excretion was at first increased but after two or three days returned to its previous level in spite of the polyuria.

Since the work of Asimoff and Estrin had not yet appeared, the work on bile was started to see if the thyroid hormone was excreted in the bile, because Kendall had reported that thyroxine was largely excreted through this route. It was found, however, in the present investigation that after thyroid feeding very little iodine was eliminated in the bile (table 2).

TABLE 2

Showing the total iodine in the bile after thyroid feeding

DOG	DAYS FED	TOTAL I_2 FED	TOTAL I_2 IN BILE
		mgm.	*mgm.*
16	5	200	3.8
17	2	80	2.1
18	2	80	0.0
19	4	80	0.0
20	7	168	0.0
21	7	154	0.0
22	1	200	7.1 (48 hrs.)

Only 3 dogs out of a series of 7 showed a trace of iodine and dog 22, which received a very large dose, excreted only 3.5 per cent of the iodine in the bile. Probably a portion of this is the result of flooding the tissues and does not represent the normal route of excretion. The results are in accord with those of Asimoff and Estrin who recovered 1.7 and 1.69 per cent of the iodine in the bile after feeding huge doses of desiccated thyroids. These results are in marked contrast to what had been reported after the injection of thyroxine and it seemed worth while to repeat the work.

In table 3 the results are shown after the intravenous injection of thyroxine into 7 dogs. The total dosage varied from 20 mgm. as a single dose to 500 mgm. in 5 injections. The question mark after the biliary iodine in dog 28 indicates that the bile collection was not complete.

The results in this series of experiments are strikingly different in that the iodine in the bile exceeds that in the urine. This is in agreement with the results of Kendall (4), Krayer (2), and Asimoff and Estrin (5). In trying to analyze the difference from thyroid feeding, it must be borne in mind that the mode of administration is different. One might suppose that the

concentration in the blood would be higher after intravenous injection than after oral administration. However, this apparently does not play an important rôle because in dog 26 only 13 mgm. of iodine were injected as thyroxine, yet the amount of iodine in the bile was nearly double that in the urine. The results of Asimoff and Estrin, showing a greater amount of iodine in the bile than in the urine after oral administration of thyroxine, are convincing. The comparatively large amount of iodine in the urine after thyroid feeding is due, in part at least, to the diiodo-tyrosine which Harington (7) has shown to be present. Oswald (8) showed many years ago that this compound was eliminated largely by the kidneys. Since

TABLE 3

Showing the iodine in the bile and urine after intravenous thyroxine

DOG	NUMBER OF INJECTIONS	TOTAL I_2 INJECTED	I_2 IN BILE	I_2 IN URINE
		mgm.	*mgm.*	*mgm.*
23	2	26.0	7.8	4.5
24	1	65.0	13.1	12.0
25	1	65.0	10.3	7.0
26	1	13.0	6.2	3.6
27	5	299.0	123.4	50.0
28	4	260.0	58.9?	59.4
29	5	325.0	149.2	58.8

TABLE 4

Showing the rate of iodine excretion after intravenous injection of 100 mgm. of thyroxine

HOUR	BILE VOLUME	I_2	HOUR	URINE VOLUME	I_2
	cc.	*mgm.*		*cc.*	*mgm.*
0–4	59	13.4			
4–8	36	6.7			
8–13	48	6.5			
13–24	47	6.3	0–24	300	6.6

thyroxine is eliminated through the bile and since very little iodine is excreted through the bile after thyroid feeding, it seems certain that free thyroxine is not liberated during the course of thyroid feeding.

Recently the rate of iodine excretion after thyroxine administration has been investigated by Krayer (2), and Asimoff and Estrin (3). This line of investigation may throw some light on the mechanism of action of the thyroid hormone. If the increase in metabolism occurs after most of the hormone has been excreted, one must conclude that the action persists after the disappearance of the stimulus, or that comparatively little of the administered dose is effective. Since Krayer's work was carried out on rats under anesthesia and Asimoff and Estrin had only two experiments

with thyroxine, it was considered worth while to extend the series. Fourteen experiments were carried out on 5 of the 7 dogs in table 3. After intravenous injection of thyroxine, the bile was collected for iodine estimations after successive periods of 4, 4, 4, and 12 hours. Since the urine contains only a small amount of iodine, analysis was made on the total 24 hour collection. Table 4 gives a typical experiment.

This dog was given 100 cc. of dog bile and fed at the time of injection. The table clearly brings out that more iodine is eliminated in the first 4 hours than in any other period of the same duration, but in contrast to Krayer's work, the majority of the iodine is not excreted so rapidly. In the 14 experiments which have been carried out, not once has more iodine been eliminated in the first 4 hours than in the next 20 hours. There are several factors different from Krayer's experiments: the species is different, his animals were anesthetized, and in some cases his dosage was smaller. In the given experiment, 39.5 mgm. of iodine appeared in the bile and urine within 24 hours. This represents over 60 per cent of the total dose. Since the basal metabolism does not rise until about 24 hours after administration of the first dose, any theory for the mechanism of action of the thyroid hormone must account for the fact that over half of the iodine in the thyroxine is eliminated before the response occurs. Further work on the rate of excretion of the hormone correlated with the increased metabolism needs to be done.

SUMMARY AND CONCLUSION

1. On feeding whole thyroids or thyroglobulin to dogs about 50 per cent of the iodine is eliminated through the urine, while very little appears in the bile.

2. After intravenous thyroxine a greater proportion of the injected iodine appears in the bile than in the urine.

3. After an intravenous injection of thyroxine, about half of the iodine is excreted in the bile and urine before a rise in metabolism occurs.

4. Since very little iodine appears in the bile after feeding large doses of dessicated thyroid, it is concluded that free thyroxine is not liberated.

The author wishes to thank Prof. A. J. Carlson for his interest and advice during the course of this work.

BIBLIOGRAPHY

(1) VON FELLENBERG, T. 1926. Ergebn. d. Physiol., xxv, 176.
(2) KRAYER, O. 1928. Arch. f. exp. Path., cxxviii, 116.
(3) ASIMOFF, G. AND E. ESTRIN. 1931. Zeitschr. f. d. ges. exp. Med., lxxvi, 380.
(4) KENDALL, E. C. 1919. Endocrinol., iii, 156.
(5) ASIMOFF, G. AND E. ESTRIN. 1931. Zeitschr. f. d. ges. exp. Med., lxxvi, 399.
(6) KENDALL, E. C. 1914. Journ. Biol. Chem., xix, 251.
(7) HARINGTON, C. R. AND S. S. RANDALL. 1929. Biochem. Journ., xxiii, 373.
(8) OSWALD, A. 1910. Zeitschr. Physiol. Chemie, lxv, 141.

THE INTERACTION OF THE CORNEAL REFLEX AND VESTIBULAR NYSTAGMUS

R. LORENTE DE NÓ

From the Central Institute for the Deaf, St. Louis, Mo.

Received for publication November 7, 1932

If the cornea of a rabbit is touched with a needle, a series of reflex responses appears; the lids and nictitating membrane will be drawn over the cornea and the eyeball will be retracted. This last effect is produced by simultaneous contraction of the retractor bulbi and the six extrinsic ocular muscles. In this paper reference will be made only to the reflex contraction of the ocular muscles. This reflex response appears to be extremely simple because in it no reciprocal innervation is involved. In the reflex arc producing it, there must be at least one internuncial neurone because there are no collaterals from the trigeminus root to the motor nuclei of the eye muscles.

Unfortunately our knowledge concerning the trigeminal pathways is so extremely limited that no statement can be made with regard to the detailed anatomical constitution of the arc between the trigeminus and the oculo-motor nuclei. It seemed desirable to determine if the whole arc, with the exception of the motoneurones, might be independent of the vestibular ocular arc. We have, therefore, attempted to analyze, by means of the corneal reflex, the state of the motoneurones during and between periods of vestibular nystagmus.

TECHNIQUE. The corneal reflex was elicited by faradic stimulation of one of the branches of the trigeminus innervating the eye lids. Generally the branch from the upper lid was used. Through a slit in the skin the nerve is exposed and a pair of suitable electrodes passed under the nerve. The possibility that spread of stimulus might cause a motor tetanus has been excluded, for if the nerve is cut central to the electrodes no contraction of the eye muscles occurs. The stimuli have been faradic, from a Porter coil, 7 to 12 cm. coil separation, two volts in the primary. The periods of stimulation are marked in the records by a signal which also opened a short circuit across the secondary. The beginning of stimulation is indicated by a downward movement of the signal tracing.

The bony horizontal canal was exposed and vestibular nystagmus produced by touching it with a cold or warm needle. The head of the rabbit was held in position tipped backward so that the external canals form an angle of about 30° with the horizontal plane.

The six ocular muscles of one eye were exposed and prepared for simultaneous recording on smoked paper by means of light isotonic levers.

All the operations have been performed under ether anesthesia.

Five experiments yielding the same result have been carried out.

RESULTS. The reflex reaction following short and long periods of trigeminal stimulation have the characteristics indicated by figure 1. If the stimulation period is short (fig. 1, *a*), the six muscles show a twitchlike contraction followed by a somewhat slower relaxation (after discharge). If the stimulation lasts for several seconds (fig. 1, *b*), the record shows a plateau that remains constant for some muscles (R. ext., R. int., O. inf.) descends slowly for others (R. sup., O. sup.) and in the other (R. inf.) first progressively ascends for several seconds and then remains nearly constant. The form of the plateau depends somewhat on which branch of the trigeminus is excited and it seems probable that the modifications in the contraction plateau represent eye movements which, in our intact animal, would move the cornea away from the stimulus.

In the plateau, especially in those muscles with rising plateaus, there appears a very rapid contraction rhythm which can scarcely be followed by our isotonic levers. This contraction rhythm might be related to the rhythm of the stimulus but, since it also appears in the intact eye when the cornea is touched with a needle, perhaps it would be more justifiable to consider it as arising in the central mechanism.

Vestibular nystagmus consists of a series of alternating contractions and relaxations; usually three muscles show slow contractions and quick relaxations and their three antagonists quick contractions and slow relaxations. The contractions are obviously due to the arrival of excitatory impulses at the motoneurones, but for the relaxations two alternative explanations may be offered. Relaxation of an ocular muscle during a period of continuous vestibular stimulation might be an expression of a resting state of its motoneurones either because the excitatory impulses were being arrested at some locus upstream from the motoneurones, or because of an active inhibition of the motoneurones which canceled the effect of excitatory impulses impingent upon them.

If during a vestibular nystagmus the trigeminus be stimulated for brief intervals, the muscles respond reflexly to the latter and the total response seems to be the result of an algebraic summation of the vestibular and trigeminal effects. In figure 2, tracings of the horizontal muscles have been reproduced. With the internal rectus where the nystagmus shows quick contractions and slow relaxations, the contraction following trigeminal stimulation seems to be the result of the summation of the excitatory impulses from both sources. Similarly with the external rectus the trigeminal impulses produce either a twitch superimposed on the slow

nystagmus contraction (fig. 2, *4*, *7*, *8*), prevent the quick relaxation of the muscle (fig. 2, *2*) or set up a small twitch (fig. 2, *3*, *5*, *9*).

But if the trigeminal stimulation is continued through several nystagmus cycles the results are different. The muscles as a rule respond to the tri-

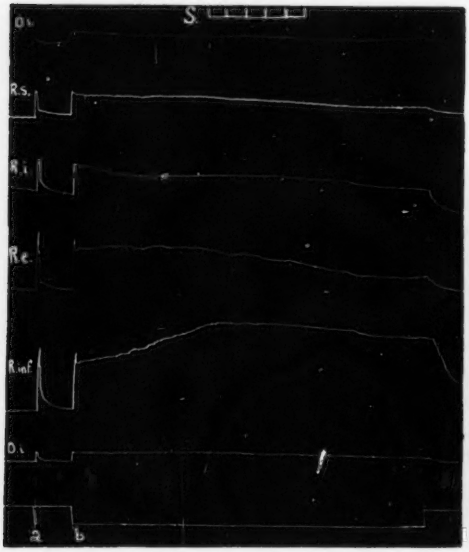


Fig. 1

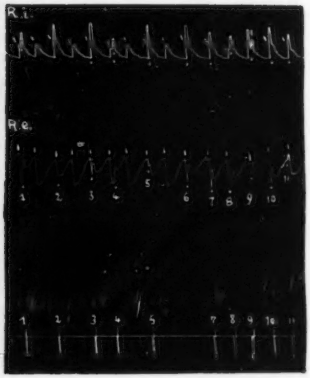


Fig. 2

Fig. 1. Reflex reactions of the six eye muscles; faradic stimulation of the nerve from the upper lid. *O. s.*, obliquus superior; *R. s.*, rectus superior; *R. i.*, rectus internus; *R. e.*, rectus externus; *R. inf.*, rectus inferior; *O. i.*, obliquus inferior, *s*, seconds; *a*, short stimulation, twitchlike contraction of the six muscles; *b*, long stimulation, the six muscles show contraction plateaus.

Fig. 2. Short faradic stimuli of the nerve from the upper lid during a vestibular nystagmus. *R. i.*, rectus internus; *R. e.*, rectus externus. *1–11*, faradic stimuli. The dots beneath the graphs indicate the beginning of the reflex reaction. The quick phases of the nystagmus are marked with dots above the graphs.

In *1*, *4*, *7*, and *8* the corneal reflex has been elicited at the beginning of slow phases of nystagmus; in *2*, *3*, *5* and *9* during quick phases, in *11* at the end of a slow phase and in *6* and *10* at the end of quick phases. In all cases the contraction of the muscles seems to be the result of an algebraic summation of vestibular and trigeminal impulses.

geminal impulses with a contraction plateau to which the nystagmus adds itself (fig. 3). When the vestibular stimulation is weak (*1*, *2*) the rhythm of the nystagmus is masked by the corneal reflex; but when the nystagmus

is strong, its rhythm again becomes apparent (*3, 10*) and there is to be seen a change of the amplitude of the nystagmus. In those muscles showing quick relaxations and slow contractions (fig. 3, *R. int., R. inf.*) the diminution of the amplitude is slight, but in those muscles showing slow relaxa-

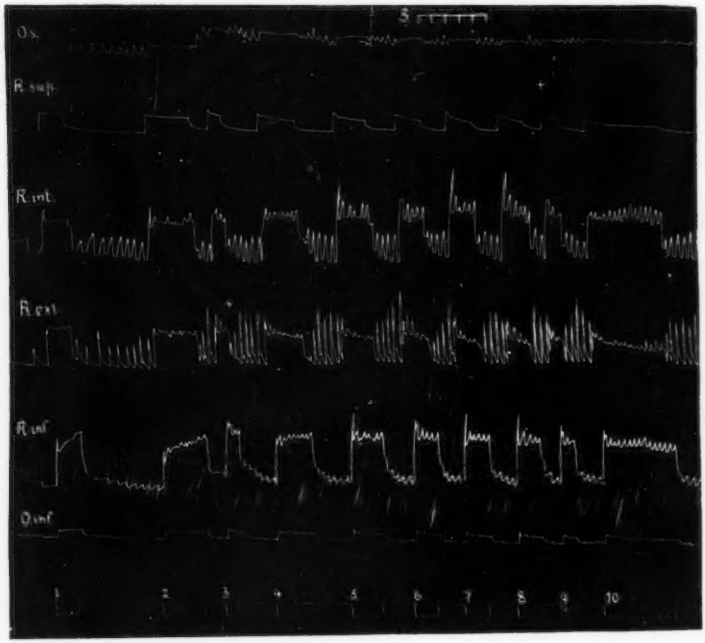


Fig. 3. Modifications of the vestubular nystagmus produced by prolonged stimulation of the nerve from the upper lid. The intensity of the nystagmus increases progressively. In *1* it is very weak and the reaction represents almost a pure corneal reflex; in *2* the rhythm of the nystagmus is still masked by that of the corneal reflex; in *4–10* the nystagmus rhythm is again apparent. Note that the amplitude of the nystagmus in those muscles showing quick contractions diminishes when the corneal reflex is produced, and that the tetanus is always less than in *1* and *2*.

tions and quick contractions (*R. ext., O. sup.*) the amplitude of the nystagmus becomes very much reduced. Furthermore, with *R. ext.* and *O. sup.*, the mean contraction level after trigeminal stimulation becomes less than before (compare intervals in fig. 3, *8, 9, 10* with *1, 2*).

These facts cannot be explained by merely assuming an algebraic summation on the motoneurones of the effects of trigeminal and vestibular impulses; because if the nystagmus relaxation should be due to an active inhibition the result of such algebraic summation would be a nystagmus

with contractions equal to the sum of the trigeminal and vestibular ones and relaxations equal to the difference, as seems to happen in figure 2; and if the nystagmus relaxation is only due to a state of rest of the motoneurones the result of the summation would be nystagmus of normal amplitude superimposed on the trigeminal contraction.

The concept of "occlusion" as developed by Sherrington and co-workers for allied spinal reflexes cannot be applied here, because occlusion has been described as taking place only when the reflex response of the motor units to one of the reflexes is maximal, and in the cases we are examining both stimulus and response were rather weak and therefore it would be very difficult to accept that a weak response is due to maximal contraction of only a few units; on the other hand the effect of occlusion would be quite different. Some motor units would be occluded (maximally excited) by the trigeminal impulses thus giving the muscle a certain contraction state to which the nystagmus (of course of less amplitude because of the number of "nystagmus units" being less) ought to be added. In no case would the corneal contraction be less than with the resting muscle. If, however, the nystagmus is somewhat stronger than in figure 3, the whole effect of the corneal reflex consists in diminishing the amplitude of the nystagmus without bringing forth an actual contraction of those muscles which have nystagmus with slow relaxations and quick contractions (fig. 4, *R. ext.*). An occlusion of the corneal reflex by the nystagmus cannot be considered because the nystagmus is an alternating reflex, and therefore can "occlude" only during the phase of contraction.

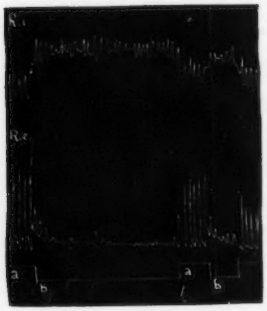


Fig. 4. The same as in figure 3 but with the nystagmus somewhat stronger. Note the *R. e.* responds to the stimulation of the trigeminus only with a diminution of the amplitude of the nystagmus; *a*, nystagmus; *b*, during faradic stimulation.

Since the assumption of an algebraic summation of the stimuli at the motoneurones proves to be insufficient it is necessary to find another explanation, and apparently the easiest is that the vestibular and trigeminal arcs have a common internuncial relay where the impulses meet before they reach the motoneurones. In this relay new impulses are produced which determine the characteristics of the motor discharge that corresponds to the combined excitation of the trigeminal and vestibular nerves.

In the vestibular system there are several neurone chains closing the reflex arc. It has been demonstrated (1), (2) that all of them work in intimate collaboration and that all are active when nystagmus is produced.

Some of these pathways running in the posterior longitudinal bundle and tractus vestibulo-mesencephalicus have nothing in common with the trigeminal system. Therefore, the interference of vestibular and trigeminal impulses must take place in other chains involving cells of the reticular substance upon which the trigeminus may exert an influence. But the impulses conducted by the p.l.b. and the tr. vest. mes. can be influenced by the trigeminal ones only when they arrive at the motoneurones. Further experiments revealed some evidence bearing on this question.

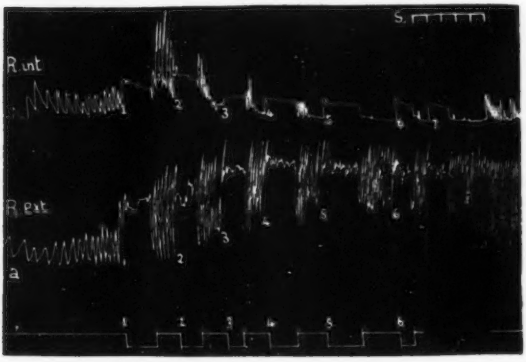


Fig. 5

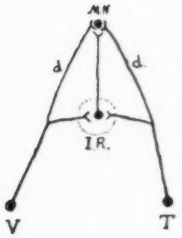


Fig. 6

Fig. 5. The corneal reflex during a strong nystagmus. The nystagmus begins in *a* and progressively increases. Between *5* and *7* the amplitude of the nystagmus of the internal rectus is very small and the muscle remains relaxed; but when the corneal reflex is elicited this muscle contracts as in *1*. At the end of the graphs the intensity of the nystagmus begins to decrease and the amplitude becomes larger.

Fig. 6. Diagram showing the simplest reflex arc that may account for the phenomena described in this paper. *V*, primary vestibular nuclei; *T*, primary trigeminal nuclei; *V. d.*, direct vestibular pathway (fasciculus longitudinalis posterior); *I. R.*, internuncial relay (reticular substance) common to both systems; *M. N.*, motor nuclei of the eye muscles; *T. d.* and *T. I. R.*, the two trigeminal pathways; *I. R.*, *M. N.*, the reticular pathways ending in the motor nuclei. The vestibular pathways are anatomically well known, the trigeminal ones have not been identified yet.

If the intensity of the nystagmus increases, both the amplitude and the frequency of nystagmus increase first, but after a certain limit is attained the amplitude decreases, although the frequency becomes still higher. Finally the amplitude becomes so small that the muscles show no nystagmus but only a steady contraction or relaxation; this effect appears first in those muscles having slow relaxations and quick contractions, which often remain relaxed while the antagonists show nystagmus of great amplitude. Such a case is that reproduced in figure 5.

If during strong vestibular stimulation the corneal reflex is elicited, every muscle reacts to it and those muscles that find themselves in a state of relaxation contract in very much the same way that they do when no nystagmus is present (fig. 5, *R. int.*). The nystagmus of the antagonistic muscles (*R. ext.*) diminishes in amplitude and becomes of irregular rhythm; the tetanus of the muscle, to which the nystagmus adds itself (8) is much higher than the contraction produced by the corneal reflex alone.

Since the existence of an internuncial relay common to the vestibular and trigeminal arcs is made likely by the previous observations, it can be provisionally accepted that the modifications of the rhythm and amplitude (*R. ext.*) have been produced in that internuncial relay. But the contraction of the relaxed muscle (*R. int.*) evidently must be explained in a different way.

The muscle (*R. int.*) responds to the corneal reflex in very much the same way that it does when no nystagmus is present. This indicates that although the muscle is relaxed the motoneurones are not inhibited but are merely in a state of rest, due to the lack of excitatory impulses. With reference to the function of the vestibular arc the following question now arises: Since the motoneurones are not inhibited there is now no reason why the impulses for the quick nystagmus contractions should remain ineffective, except that they do not reach the motoneurones. Experiments bearing on this question have been published elsewhere (3).

On the other hand, the fact that the relaxed internal rectus (fig. 5) shows an apparently unmodified corneal reflex, although the reflex reaction of the external rectus proves the existence of an interference of the trigeminal and the vestibular impulses, points strongly to the existence in the trigeminal arc of another direct path through which the impulses may reach the motoneurones independently of the vestibular impulses.

In other words, the trigeminal arc must be composed at least of two neurone chains, one partly in common with the vestibular arc where interference of the stimuli takes place and another more simply built through which the impulses may arrive directly at the motoneurones. Figure 6 indicates diagrammatically how the trigeminal pathways can be thought of. According to this diagram the vestibular and the trigeminal impulses meet in two places, the common internuncial relay *IR* and the motoneurones *MN*, and besides the *IR* impulses will meet at the motoneurone both the *Vd.* and *Td.* ones.

Discussion. The corneal reflex in spite of its apparent simplicity undoubtedly requires the activity of a complicated mechanism similar to that of the vestibular arc where several neurone chains all working at the same time have been demonstrated. It is now interesting to note that the corneal reflex and the spinal flexor reflex, from certain points of view, are very much alike.

No inhibition of the motoneurones during nystagmus has been demonstrated in spite of the existence of relaxations reciprocal to contractions of antagonistic muscles. The relaxation seems to be due merely to lack of excitatory impulses arriving at the motoneurones. If an active inhibition of neurones take place, it must occur in some internuncial relay further upstream.

SUMMARY

The corneal reflex produced by faradic excitation of the trigeminal branch innervating the upper lid has been pitted against a pre-existent vestibular nystagmus. The results obtained can not be explained simply by the assumption of an algebraic summation of the impulses arriving at the motoneurones. The only explanation seems to be that the trigeminal arc is composed of at least two different neurone chains, one including internuncial relays partly common with the vestibular arc. No evidence of an active inhibition of motoneurones in the case of vestibular reflexes has been found.

BIBLIOGRAPHY

(1) Lorente de Nó, R. Die Labyrinthreflexe auf die Augenmuskeln, etc. Urban and Schwarzenberg, Wien, 1928 (also in Monatschrift f. Ohrenheilk. 1927).
(2) Lorente de Nó, R. Ergebn. d. Physiol., 1931, xxxii.
(3) Lorente de Nó, R. The vestibular reflex arc (in publication).
(4) Cooper, S., D. F. Denny Brown and C. S. Sherrington. Proc. Roy. Soc. 1927, ci B, 262.
(5) Sherrington, C. S. Proc. Roy. Soc., 1929, cv B, 332; Brain, liv, 1.

STUDIES ON THE CORONARY CIRCULATION

II. The Absorption of Lactic Acid and Glucose and the Gaseous Exchange of Heart Muscle[1]

DANIEL A. McGINTY and A. T. MILLER, Jr.

From the T. T. Fishburne Laboratory of Physiology, Emory University School of Medicine, Emory University, Ga.

Received for publication November 12, 1932

Himwich, Koskoff and Nahum (1928) have shown that lactic acid is absorbed by the heart from the blood in the coronary system. This observation was confirmed and extended to show that absorption took place in the heart muscle even under adverse circulatory conditions (McGinty, 1931). Since it has been established that lactic acid is taken up by the heart muscle, the manner of its disposal becomes a matter of interest. In previous investigations as to the nature of the carbohydrate oxidized in the course of metabolism of cardiac muscle, attention generally has been centered on glucose not only because results have been interpreted as indicating that this substance is absorbed by heart muscle (Locke and Rosenheim, 1907) (Knowlton and Starling, 1912) (Maclean and Smedley, 1913) (Cruickshank and Patterson, 1913) (Underhill and Prince, 1914) but also because the gaseous exchange of the heart has been correlated with glucose utilization (Rohde, 1910) (Gayda, 1911) (Evans, 1912) (Starling and Evans, 1914–15) (Visscher and Mulder, 1930). However, the magnitude of lactic acid absorption is fully comparable to the values for glucose absorption as reported. As other investigators (Patterson and Starling, 1913) (Clark, 1916) have failed to obtain direct evidence for glucose utilization by the heart under similar conditions, considerable doubt exists concerning the part played by glucose in the metabolism of this organ. In an effort to clarify this matter and to determine the fate of lactic acid absorbed by the heart, the question of carbohydrate utilization and gaseous exchange has been reopened.

In previous studies two general methods have been used. In the first, an isolated surviving heart was perfused with oxygenated Ringer-glucose solutions. Conclusions as to glucose absorption were based on analyses of the perfusion fluid at intervals during the experiment. In the second method, the heart-lung preparation was employed. Evidence for glucose

[1] Aided by a grant from the Committee on Scientific Research of the American Medical Association.

utilization was obtained from periodic examinations of the blood in the system. Various modifications of the heart-lung preparation have been used for study of the gaseous exchange of the heart.

In the present study, two procedures were followed. In the first, an isolated beating dog heart was perfused through a cannula placed in the aorta by blood from the carotid artery of a donor dog. This method insured perfusion of the isolated heart at a uniform rate by blood which differs from normal blood only in that the donor animal was anesthetized and its blood rendered non-coagulable with heparin. This procedure in common with any method of isolated heart perfusion, and to a considerable extent in the heart-lung preparation, has the disadvantage that the isolated heart may exhibit an abnormal metabolism because of the interrupted circulation of blood during its preparation.

For the second series of experiments, the beating heart, *in situ*, was used (McGinty, 1931). This method entails practically no disturbance to the heart and the circulation through the coronary vessels is uninterrupted.

In either procedure, samples of arterial and of coronary venous blood were withdrawn simultaneously and analyzed for lactic acid, glucose and oxygen and carbon dioxide content. Arterial-venous differences in these blood components were taken as criteria of absorption by the heart muscle or elimination from it. Although these differences may be small with a single passage of blood through the coronary capillaries, it became apparent that with a sufficient number of analyses, the procedure has outstanding advantages over the former methods used in studying the carbohydrate and gaseous metabolism of this organ. In addition, the procedure is justified by the greater accuracy of analytical methods now available.

EXPERIMENTAL. *First method.* The donor dogs of 8 to 12 kgm. body weight were anesthetized with morphine (5 mgm. morphine sulfate per kgm.) and sodium amytal (35–40 mgm. per kgm.). Cannulae were placed into a carotid artery and femoral vein and heparin was injected. Larger dogs of 12 to 15 kgm. body weight, lightly anesthetized with morphine and ether, were bled rapidly from a carotid artery. Just before opening the chest and removing the heart, several hundred cubic centimeters of warmed Ringer-Locke solution were introduced into an external jugular vein in order to prevent accidental clotting in the coronary vessels. Glass cannulae were tied into the aorta and coronary sinus. With minimal delay, perfusion was started by connecting the arterial cannula of the donor with the aortic cannula of the isolated heart. Blood pressure at the aortic cannula was recorded. The coronary venous blood was collected, its rate of flow recorded and returned along with other blood which escaped from the heart, at constant venous pressure to the donor dog. Additional blood was added at intervals to replace that removed during sampling. The temperature of the donor was maintained by heating coils so that the blood

entering the heart remained at 35 to 37°C. Drying and cooling were prevented by superficial shielding of the heart by gauze. Such preparations beat well for several hours although the data herewith recorded were obtained in a maximal period of 95 minutes.

Second method. This was essentially the same as that described in an earlier paper (McGinty, 1931). Morphine and sodium amytal were used in minimal amounts. During artificial respiration, the chest was opened in the midline and a ligature placed around the coronary sinus at the opening into the right auricle. Heparin was introduced and a cannula tied first into the right auricle with a purse string suture and lastly into the sinus. Every precaution was observed to minimize obstruction to the flow of blood from veins entering the sinus and from the sinus into the cannula. The sinus outflow was measured, recorded and returned to the animal.

Arterial and coronary venous blood samples were taken within a few seconds of each other and at intervals of five to nine minutes during the experiment. These were transferred immediately to narrow glass test tubes and stored under heavy oil at 0°C. The blood samples were analysed in pairs, in from one to five hours, for carbon dioxide and oxygen content (Van Slyke and Neill, 1924). Deproteinization was carried out within two hours of sampling according to the method of Somogyi (1930) and the filtrate stored at 0°C. Subsequently, a part of the filtrate was analysed in triplicate for lactic acid after removal of glucose (Friedemann, Cotonio and Shaffer, 1927). Another portion was analysed in triplicate for glucose using a modified Shaffer-Hartmann procedure. The utmost care in sampling and analytical treatment was observed since errors of duplicate analyses approach the order of magnitude of arterial-venous differences in concentration. In the case of glucose, the error of determination is greater than the average arterial-venous difference.

RESULTS. Five experiments were performed on the perfused isolated beating heart. In four of these, twelve pairs of blood samples were withdrawn at fairly regular intervals over perfusion periods of 60 to 90 minutes. In one experiment of 30 minutes' duration, five pairs of samples were taken. Lactic acid, glucose and oxygen absorption and carbon dioxide production were calculated from the arterial-venous difference, as well as from the heart weight and the coronary sinus volume flow of blood assuming that this amounted to 60 per cent of the total volume of blood passing through the coronary system (Markwalder and Starling, 1913) (Anrep, Blalock and Hammouda, 1929). The coronary flow was calculated for a period beginning one to two minutes before sampling and ending one to two minutes after sampling. The average results for an entire perfusion period are given in table 1. In the five observations, lactic acid absorption varied from 0.4 to 2.5 mgm. per gram heart muscle per hour, with a mean of 1.5 mgm. Glucose absorption ranged from zero to 0.4 mgm. with an average of 0.2 mgm. per gram per hour.

The mean oxygen absorption rate for five experiments was 3.5 cc. per gram heart per hour. This value agrees closely with that of Rohde for the cat, Gayda for the rabbit heart perfused with Ringer-Locke solutions, and with the oxygen absorption rates of Evans (1912) (1914) and Starling and

TABLE 1

Lactic acid and glucose absorption and gaseous exchange of perfused isolated beating dog heart

EXPERIMENT NUMBER	DURATION OF PERFUSION	NUMBER OF SAMPLES	LACTIC ACID ABSORPTION	GLUCOSE ABSORPTION	OXYGEN ABSORPTION	VOLUME FLOW	R.Q.	ARTERIAL LACTIC ACID RANGE	ARTERIAL GLUCOSE RANGE
			Per gram heart per hour						
	minutes		*mgm.*	*mgm.*	*cc.*	*cc.*		*mgm. per cent*	*mgm. per cent*
1	60	12	2.3	0.0	3.9	48	0.79	18.1–27.5	129.2– 98.6
2	85	12	1.0	0.2	2.7	49	0.55	19.3–29.3	97.7– 77.5
3	90	12	2.5	0.4	2.4	55	0.82	19.6–25.5	94.1– 73.5
4	30	5	0.4	0.4	3.0	42	0.61	11.8–19.5	119.2–104.7
5	60	12	1.2	0.01	5.7	42	0.51	27.8–38.7	142.1–130.9
Mean.....			1.5	0.2	3.5	47			

TABLE 2

Lactic acid and glucose absorption and gaseous exchange of dog heart in situ

EXPERIMENT NUMBER	DURATION OF PERFUSION	NUMBER OF SAMPLES	LACTIC ACID ABSORPTION	GLUCOSE ABSORPTION	OXYGEN ABSORPTION	VOLUME FLOW	R.Q.	ARTERIAL LACTIC ACID RANGE	ARTERIAL GLUCOSE RANGE
			Per gram heart per hour						
	minutes		*mgm.*	*mgm.*	*cc.*	*cc.*		*mgm. per cent*	*mgm. per cent*
6	65	12	3.4	0.25	4.9	58	0.76	63.0–131.5	114.1– 22.0
7	80	12	1.6	0.2	4.6	31	0.70	11.4– 20.3	150.0–130.2
8	75	12	0.6	0.0	5.1	58	0.57	12.5– 29.8	127.0–154.5
9	80	9	3.4	1.2	4.8	42	0.80	33.3– 53.6	93.2–141.0
10	30	4	4.6	0.2	5.4	50	0.77	21.6– 32.9	107.9– 96.1
11	35	6	5.9	0.7	6.5	65	0.88	35.2– 42.9	97.9–103.7
12	70	10	1.7	0.17	4.8	40	0.72	16.7– 37.8	179.4–194.1
Mean.....			3.1	0.39	5.1	49			

Evans in dogs and cats using the heart-lung preparation. The coronary blood flows lie within the limits of 40 to 60 cc. which according to Anrep (1926), represent the magnitude of the cardiac circulation in the early stages of experiments using the heart-lung apparatus. In two experiments, the average respiratory quotients were above 0.70 and in three experiments

below this level. The lower R.Q.s correspond to the lower rates of absorption of lactic acid.

In the group of experiments in which the heart remained intact within the open thorax, seven observations were carried out for periods of 30 to 80 minutes. Blood samples were withdrawn as before at regular intervals. The results calculated as in the first series are condensed in table 2. The mean lactic acid absorption of all observations was 3.1 mgm. while glucose absorption was 0.39 mgm. per gram per hour. Average oxygen absorption was 5.1 cc. and mean coronary flow was 49 cc. per gram per hour. Again one sees a rough parallelism between the R.Q. and the average rate of lactic acid absorption for each experiment. In this group, an R.Q. below 0.70 occurred in but one experiment. The last two columns of tables 1 and 2 show the range of lactic acid and glucose concentrations in arterial blood during the periods of perfusion.

DISCUSSION. The results of these experiments are in harmony with those of other investigators in some respects but at variance in others. Attention already has been called to the close agreement between the rate of oxygen absorption in the experiments on the isolated heart and the findings obtained with the heart-lung preparation. Oxygen absorption in the experiments on the heart *in situ* is higher as would be expected since alterations or interruptions in the activity of the heart were negligible. It corresponds exactly with the values calculated from the results of Bayliss, Müller and Starling (1928). The volume flow of blood in the coronary circulation likewise agrees with that obtained with the heart-lung preparation with a possible exception in experiment 7. Here the rate was lower than usual although the heart appeared to be in good condition during the 80 minutes' perfusion.

An examination of these results using the isolated heart as well as the heart normally perfused, indicates that under the prevailing conditions little or no glucose is absorbed and that the absorption of lactic acid is sufficiently great to account for the generally accepted quantity of material oxidized as carbohydrate. These findings are so different from those of other workers that it becomes necessary to consider the experimental procedures followed as a possible explanation of the differences in results.

In the experiments of Locke and Rosenheim, Rohde, Gayda, Maclean and Smedley, Cruickshank and Patterson and Underhill and Prince, isolated rabbit, cat and dog hearts were perfused for prolonged periods with Ringer-glucose solutions. It was inferred that glucose utilization took place because of its disappearance from the perfusion fluids. Clark, however, has demonstrated that there is little or no loss of glucose from saline perfusates under similar conditions if rigid precautions are taken to exclude bacteria. Perfusion of the isolated heart by glucose saline solutions as a means of demonstrating glucose utilization by this organ is open to another

criticism. It is not likely that such fluids can convey to the heart but a part of the oxygen that it normally requires so that its oxidative mechanism becomes abnormal. Under these anoxemic conditions lactic acid is not absorbed readily (McGinty). It is conceivable that with the failure of lactic acid absorption, glucose is taken up by cardiac muscle either for oxidation or, as Visscher and Mulder have suggested, to supply carbohydrate for the excessive lactic acid formation taking place.

Turning to those investigators who used the heart-lung preparation (Knowlton and Starling; Cruickshank and Patterson; Bayliss, Müller and Starling; Visscher and Mulder), it may be found that their procedures are open also to adverse criticism despite the use of blood instead of artificial blood solutions for perfusion. While the supply of oxygen for the heart is adequate, there is little or no evidence that the apparent glucose disappearance may not have been due in part, at least, to bacterial action. When Patterson and Starling employed strict aseptic precautions against bacterial decomposition of glucose, they were unable to demonstrate a loss of glucose in heart-lung perfusates. They interpreted these results, however, not to the absence of bacteria but to other causes. Another mechanism which might explain the apparent loss of glucose from blood in the heart-lung system, is glycolysis in the blood itself. It may be calculated from the experiments, *in vitro*, of Häusler (1927) at high blood sugar concentrations and of Braunstein (1931) at normal levels that the reaction glucose → lactic acid may yield lactic acid at a sufficiently high rate to account for that absorbed by the heart.

It appears, therefore, that there is no direct unassailable evidence either from the isolated heart perfusion experiments or from the heart-lung observations that glucose is absorbed by the normal heart. This conclusion is in line with the failure of glucose absorption in the experiments herein reported and with the fact that the absorption of lactic acid will account quantitatively for the carbohydrate said to be oxidized by heart muscle.

However, before one can accept the view that blood lactic acid serves as the principal source of carbohydrate for oxidation in the heart, it will be necessary to determine the status of the glycogen stores of this organ since absorbed lactic acid may be converted to glycogen and stored as such during the periods of perfusion. That heart glycogen can be mobilized readily is demonstrated by its rapid disappearacce when the heart is excised or by the fact that its concentration in the heart falls during perfusion when no other source of carbohydrate is available (Witting, Markowitz and Mann, 1930). However, Cruickshank (1913) found that the glycogen stores of the heart were not depleted in the heart-lung preparation when the glucose content of the blood was normal. This observation was confirmed by Starling and Evans and more recently by Visscher and Mulder. Cruickshank found also that heart glycogen increased whenever there

occurred a prolonged rise in the blood sugar level. Since the concentration of blood glucose in these experiments remained within the normal range, it is unlikely from the foregoing considerations that a material change in the glycogen content of the heart took place.

Absorbed lactic acid therefore, must be oxidized directly, or if it is converted to glycogen, it is changed to glucose for oxidation at the same rate. Himwich (1932) believes that the heart absorbed lactic acid but denies that it is able to oxidize this substance directly. He bases his views on the low respiratory quotients of perfused diabetic dog hearts which should not occur unless one holds that insulin is necessary for the oxidation of lactic acid. Soskin (1930), however, has demonstrated that low R.Q.s in the diabetic dog are not associated necessarily with failure of oxidation of carbohydrate. His reasoning applies equally well to the R.Q. of an isolated organ whether receiving insulin or not. One cannot overlook the possibility of interconversion of fat to carbohydrate, of carbohydrate to fat, of protein to carbohydrate and of many intermediate metabolic reactions in the isolated organ if such processes occur in the animal as a whole. As an illustration, pyruvic acid may form from lactic acid. This reaction which is known to occur in tissues under certain conditions, requires oxygen but no carbon dioxide is formed. If this reaction occurred in the heart under the prevailing conditions, the degree of lowering of the R.Q. would depend on the proportion of absorbed or formed lactic acid undergoing this change. The pyruvic acid may pass into the venous blood and be disposed of elsewhere in the body. It would not be analysed as lactic acid in this blood (Clausen, 1922). The R.Q. of the heart may be considered as a resultant of intermediate reactions as well as completed oxidations and the low quotients obtained in the experiments on the isolated heart do not indicate necessarily that lactic acid or glucose is not oxidized. The higher R.Q.s in the experiments *in situ*, where it is probable that oxidations in the heart are more like those that take place in this organ in the intact animal, likewise cannot be considered as indices to the nature of the food oxidized. However, the parallelism between the height of the quotient and the quantity of lactic acid absorbed, suggests that this substance is utilized. Whether this occurs directly or not, cannot be stated from this fact alone.

The quantity of oxygen required for the oxidation of absorbed lactic acid as calculated from the experiments *in situ*, amounts to 44 per cent of the total oxygen taken up by the heart. Visscher and Mulder calculated that oxidation of carbohydrate in the heart required not more than 60 per cent and in some cases less than 25 per cent of the total oxygen intake.

SUMMARY AND CONCLUSIONS

With the accumulation of data showing that lactic acid is absorbed by the heart of the dog in amounts corresponding to the quantity of material

generally accepted as being oxidized as carbohydrate, it was found necessary to reopen the study of oxidations in cardiac muscle and to investigate the manner of disposal of absorbed lactic acid.

Two methods were employed: first, perfusion of an isolated beating heart by arterial blood from a donor dog, and second, perfusion of the beating heart *in situ*. In either method, simultaneous samples of ingoing arterial blood and outcoming coronary venous blood were analysed for lactic acid, glucose and oxygen and carbon dioxide content. Coronary volume flow of blood was recorded. Perfusions were carried out for 30 to 90 minute periods, blood samples being taken at five to nine minute intervals.

In five experiments on perfusion of the isolated heart with a total of 53 pairs of blood samples, the average lactic acid absorption was 1.5 mgm., glucose absorption 0.2 mgm. and oxygen absorption 3.5 cc. per gram of heart muscle per hour. Blood flow amounted to 42 to 55 cc. per gram per hour.

In seven experiments with the heart intact within the open thorax, in 65 samples, average lactic acid absorption amounted to 3.1 mgm., glucose 0.39 mgm. and oxygen intake 5.1 cc. per gram heart per hour. Mean coronary flow was 49 cc.

In the first group of observations, the respiratory quotient was below 0.70 in three and above 0.70 in two. In the latter group, an R.Q. below 0.70 occurred in but one experiment.

The results indicate that glucose is not absorbed at all or absorbed in but small amounts while lactic acid is removed from the coronary arterial blood in quantities which suggest its utilization as the carbohydrate fuel of the heart.

The results of other workers who interpreted the loss of glucose from perfusion fluids as indicating its absorption by the heart are criticized on the basis of failure to exclude decomposition of glucose as a result of bacterial action. This has been shown to be an important source of error in perfusion procedures. Furthermore, those who worked with blood as a nutrient fluid have failed to consider glycolysis in blood which takes place with significant rapidity.

The status of the glycogen stores of the heart was not investigated although a review of literature indicates that under the experimental conditions which prevailed, the glycogen content of the heart was neither increased nor drawn upon for carbohydrate oxidation.

Significance of the respiratory quotient as an index to the fuel of the heart is criticized. It is believed that this quotient is of questionable value in determining the nature of oxidations in isolated organ perfusion. Under more normal experimental conditions, it may assume greater significance.

BIBLIOGRAPHY

ANREP, G. V. 1926. Physiol. Rev., vi, 596.

ANREP, G. V., A. BLALOCK AND M. HAMMOUDA. 1929. Journ. Physiol., lxvii, 87.

BAYLISS, L. E., E. A. MÜLLER AND E. H. STARLING. 1928. Journ. Physiol., lxv, 33.

BRAUNSTEIN, A. E. 1931. Biochem. Zeitschr., ccxl, 68.

CLARK, A. H. 1916. Journ. Exp. Med., xxiv, 621.

CLAUSEN, S. W. 1922. Journ. Biol. Chem., lii, 263.

CRUICKSHANK, E. W. H. 1913. Journ. Physiol., xlvii, 1.

CRUICKSHANK, E. W. H. AND S. W. PATTERSON. 1913. Journ. Physiol., xlvii, 381.

EVANS, C. L. 1912. Journ. Physiol., xlv., 213.
1914. Ibid., xlvii, 407.

FRIEDEMANN, T. E., M. COTONIO AND P. A. SHAFFER. 1927. Journ. Biol. Chem., lxxiii, 335.

GAYDA, T. 1911. Zeitschr. allg. Physiol., xiii, 1.

HÄUSLER, H. 1927. Pflüger's Arch., ccxvii, 134.

HIMWICH, H. E. 1932. Yale Journ. Biol. and Med., iv, 259.

HIMWICH, H. E., Y. D. KOSKOFF AND L. H. NAHUM. 1928. Proc. Soc. Exp. Biol. and Med., xxv, 347.

KNOWLTON, F. P. AND E. H. STARLING. 1912. Journ. Physiol., xlv, 146.

LOCKE, F. D. AND O. ROSENHEIM. 1907. Journ. Physiol., xxxvi, 205.

MACLEAN, H. AND I. SMEDLEY. 1913. Journ. Physiol., xlv, 470.

MARKWALDER, J. AND E. H. STARLING. 1913. Journ. Physiol., xlvii, 275.

McGINTY, D. A. 1931. This Journal, xcviii, 244.

PATTERSON, S. W. AND E. H. STARLING. 1913. Journ. Physiol., xlvii, 137.

ROHDE, E. 1910. Zeitschr. Physiol. Chem., lxviii, 181.

SOMOGYI, M. 1930. Journ. Biol. Chem., lxxxvi, 655.

SOSKIN, S. 1930. Journ. Nutr., iii, 99.

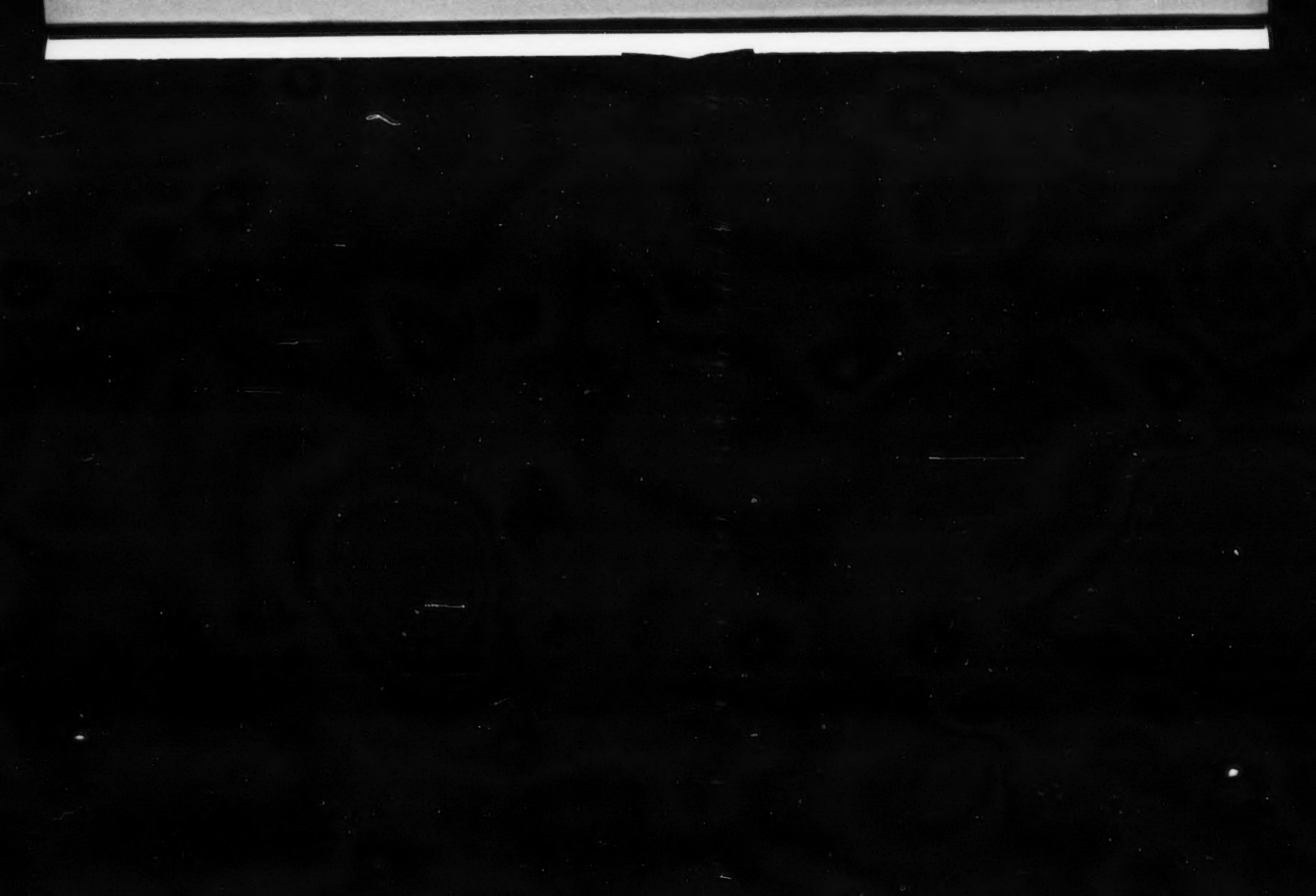
STARLING, E. H. AND C. L. EVANS. 1914–15. Journ. Physiol., xlix, 67.

UNDERHILL, F. P. AND A. L. PRINCE. 1914. Journ. Biol. Chem., xvii, 299.

VAN SLYKE, D. D. AND J. M. NEILL. 1924. Journ. Biol. Chem., lxi, 297.

VISSCHER, M. B. AND A. G. MULDER. 1930. This Journal, xciv, 630.

WITTING, V., J. MARKOWITZ AND F. C. MANN. 1930. This Journal, xciv, 35.



The American Journal of Physiology is issued monthly by the American Physiological Society under the direction of the Council of the Society. From three to four volumes, each of about seven hundred and twenty pages are published yearly. The subscription price per volume in the United States and Canada is $7.50; in other countries, $8.00.

Manuscripts submitted for publication may be sent to any member of the Council or to the Managing Editor, Dr. D. R. Hooker, 19 W. Chase Street, Baltimore, Md. They should be packed flat, carefully protected from injury, and insured.

Each article should conclude with a brief summary of results suited to the needs of abstract journals.

References to literature should accord with the following arrangement. In the text author's name is to be followed by the year date of the publication in parenthesis, thus Smith (1911). If the author's name is not mentioned in the text, the parenthesis is to contain that name as well as the date, thus (Smith, 1911). If reference is made to more than one publication of an author in a given year, the year date is to be followed by the letters a, b, c, etc., thus Smith (1911a). The bibliography at the end of the article is to list all text references according to authors' names, with initials, alphabetically arranged, thus, Smith, J. C. 1911. Journ. Biol. Chem., xii, 306.

Authors are especially urged to verify personally the accuracy of their bibliographies by reference to the original sources, since inadvertent errors cause readers great inconvenience.

All figures for illustration must be submitted in such form as to admit of photographic reproduction without retouching or redrawing. Marginal letters or figures cannot be set in type and must, therefore, be drawn in India ink by the author and regarded as part of the figure. Unless specific instructions are given by authors, the printer will be allowed to use his judgment in determining the amount of reduction on all figures. The JOURNAL cannot accept illustrations which, when reproduced for printing, exceed the size of a JOURNAL page ($4\frac{1}{2} \times 7\frac{1}{2}$ inches), unless the author is prepared to pay the cost of an insert page.

Plotted curves must be drawn in India ink on **blue lined coördinated paper.** Blue ink will not show in reproduction, so that all guide lines, letters, etc., must be in India ink.

Best results are obtained when such figures are made proportional to the shape of the JOURNAL page so that, when reduced, they will fit without undue loss of space.

PHYSIOLOGICAL REVIEWS

PHYSIOLOGICAL REVIEWS is owned and controlled by the American Physiological Society. It is edited by a board appointed by the society consisting of:

A. J. CARLSON, Chicago — H. S. GASSER, New York
LEO LOEB, St. Louis — W. R. BLOOR, Rochester
J. J. R. MACLEOD, Aberdeen, Scotland — C. D. LEAKE, San Francisco
D. R. HOOKER, Managing Editor, Baltimore

The Editorial Board selects the subjects and authors of all articles which are published, the aim being to provide concise but comprehensive reviews of the recent literature and present status of various subjects in Physiology, using this term in a broad sense to include Bio-chemistry, Bio-physics, Experimental Pharmacology and Experimental Pathology. Each volume will consist of from sixteen to twenty articles, making a total of about five hundred pages per volume. The numbers will be issued quarterly in January, April, July and October of each year.

Subscriptions will be received in advance only. The subscription price is:

$6.00 per volume in the United States, net postpaid
$6.25 per volume in Canada, net postpaid
$6.50 per volume elsewhere, net postpaid
$2.50 per single number, net postpaid

Subscriptions should be sent to

D. R. HOOKER, *Managing Editor*
19 W. CHASE STREET, BALTIMORE, MD.

Science outruns the Bookmaker—

PHYSIOLOGICAL ZOOLOGY

a quarterly devoted to recording current researches

In the October issue:

The Intracellular Digestion of Thymus Nucleoprotein in Triclad Flatworms, by Edward G. Kelley
Studies on the Sexual Cycle in Birds. V, by Thomas Hume Bissonnette
The Action of Ultraviolet Radiation on Limax flavus Linnaeus, by Emmett B. Carmichael
Placental Permeability to Insulin in the Albino Rat, by Edward L. Corsey
The Sensitivity of the Legs of Certain Calliphoridae to Saccharose, by Selma Crow
Available Food and Crowding as Factors Influencing the Sex of Moina macrocopa, by C. A. Stuart, Juanita Tallman, and H. J. Cooper

$7.50 a year **Single copy, $2.25**

THE UNIVERSITY OF CHICAGO PRESS

5750 Ellis Avenue **Chicago, Illinois**

PHYSIOLOGICAL REVIEWS

Contents of Volume XIII, 1933

F. C. Koch: The Male Sex Hormone
C. M. Gruber: Autonomic Control of Genito-Urinary Organs
A. H. Steinhaus: The Physiological Effects of Exercise
C. P. Richter: The Psycho-Galvanic Reflex
I. deB. Daly: The Vascular System of the Lungs
R. K. Burns: Experimental Transformations of Sex in Vertebrates
E. S. G. Barron: Cellular Respiration
A. Hurtado: Adaptation to High Altitude
G. W. Corner: Hormonic Control of Mammary Secretion
E. G. Wever: Mechanism of Sound Perception in the Cochlea
W. A. Jacobs: Chemistry of Digitaloid Glucosides
H. S. Liddell: Recent Contributions to the Physiology of the Conditioned Reflex
J. G. Dusser de Barenne: The Site of Action of Strychnine in the Central Nervous System
J. J. Morton and W. J. M. Scott: Sympathetic Vasoconstriction in Normal and Pathological Arteries of the Extremities
C. E. Bills: Physiology of Sterols and the Influence of Light on Sterols
C. E. Brunton: The Acid Output of the Kidney and the So-called Alkaline Tide
H. E. Jordan: Histology of the Muscle Fibre in Relation to Contraction
A. C. Ivy: Physiology of the Gall Bladder
E. F. Adolph: The Metabolism and Distribution of Water in Body and Tissues
F. J. McClure: A Review of Fluorine and its Physiological Effects
G. F. Marrian: Recent Advances in the Chemistry and Biological Assay of Oestrin
S. Mudd, Lucke and McCutcheon: Experimental Studies on Phagocytosis
J. Murphy: Sarcomata Due to Filterable Agents
F. B. Hanson: Experimentally Induced Mutations
R. G. Garry: The Physiology of the Rectum and Lower End of the Gut
W. S. Duke-Elder: Applications of Physical Chemistry in the Study of Intra-Ocular Pressure
K. S. Lashley: Integrative Functions of the Cerebral Cortex
H. E. Roaf: Colour Vision
L. H. Weed: Positional Adjustments of the Pressure of the Cerebrospinal Fluid

Subscriptions may be sent to D. R. Hooker, Managing Editor, 19 W. Chase Street, Baltimore, Md. $6.00 per volume, domestic. $6.50 per volume, foreign.

THE AMERICAN JOURNAL OF PHYSIOLOGY

(*Contents continued from cover page 1*)

The Excretion of Iodine in Experimental Hyperthyroidism. *B. O. Barnes*..... 699
The Interaction of the Corneal Reflex and Vestibular Nystagmus. *R. Lorente de N6*..... 704
Studies on the Coronary Circulation. II. The Absorption of Lactic Acid and Glucose and the Gaseous Exchange of Heart Muscle. *Daniel A. McGinty and A. T. Milier, Jr.*..... 712
Index..... 721